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Petria Thompson April 15, 2012

Potential Cross-Talk the between Aryl Hydrocarbon Receptor (AHR) and the Constitutive Androstane Receptor (CAR)

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Abstract

Potential Cross-Talk between the Aryl Hydrocarbon Receptor (AHR) and the Constitutive Androstane Receptor (CAR)

By Petria S. Thompson

Cytochrome P450s (CYPs) are a major class of hemoproteins responsible for the oxidation of endogenous and exogenous compounds as well as the metabolism of over 70% of commercially available drugs. The induction of different CYPs is regulated by several transcription factors. The aryl hydrocarbon receptor (AHR), the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR) are three ligand-activated transcription factors that regulate the transcription of cytochrome P450s—CYP1A, CYP2B, and CYP3A respectively. While there has been extensive evidence of the cross-talk between CAR and PXR there has been very limited proof of cross-talk between AHR and CAR. Using combinations of AHR, CAR, and PXR agonists in primary rat hepatocytes we studied gene expression at the RNA and protein level for CYP1A, CYP2B, and CYP3A. This work provides evidence of an AHR and CAR cross-talk phenomenon. Specifically, AHR activation suppresses phenobarbital-mediated induction of CYP2B at the RNA and protein level with a significant attenuation at 48 hours. This down-regulation of CY2B occurred with two structurally different AHR agonists, beta-napthoflavone (β-NF) and 3-methylcholanthrene (3MC). Additionally, the attenuation of CYP2B expression is via an AHR: CAR interaction and not an AHR: PXR interaction. Attempts to chemically inhibit AHR activation with the AHR antagonist 6, 2, 4-trimethoxyflavone (TMF) were futile, suggesting ligand specific antagonism. When investigating CAR translocation during exposure to AHR agonists and PB, initial experiments showed no change in the amount of nuclear CAR. However, future studies are needed to further elucidate CAR translocation during co-treatment. Evidence gained from this study as well as other studies looking at cross-talk and/or repression of nuclear factors can be used to speculate how AHR inhibits CAR's regulation of CYP2B. Finally, understanding any potential cross-talk between AHR and CAR could reveal any potential for drug-drug interactions as well as further our understanding of the regulation of cytochrome P450s via nuclear factors.

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1. Introduction

1.1 Cytochrome P450 and Xenobiotic Clearance

From the food we eat, to the air we breathe, and the drugs we take—the human body is constantly being exposed to a myriad of xenobiotic or exogenous substances. While sensing and clearing exogenous compounds is important, our body must also maintain physiological homeostasis to keep the levels of endogenous chemicals such as hormones in balance. The detoxification and elimination of these compounds is mediated through phase I and phase II metabolizing enzymes (Guengerich F. , 1991) (King, 2009). Phase I enzymes primarily introduce new polar groups via oxidation, modify functional groups to become more polar via reduction, or unmask existing polar functional groups via hydrolysis (Guengerich F. , 1991) (King, 2009). Phase II enzymes are typically involved in conjugation reactions with the addition of a new group (King, 2009). Overall, phase I enzymes increase the polarity of compounds leading to excretion or subsequent biotransformation by phase II enzymes (Guengerich F. , 1991).

Cytochrome P450s (CYPs), are phase I metabolizing enzymes that are primarily responsible for the biotransformation and elimination of xenobiotics (e.g. environmental pollutants, drugs) and the metabolism of endogenous compounds (e.g. steroids, fatty acids, cholesterol). Cytochrome P450s are a superfamily of heme-containing monoxygenases that are found primarily in the lipid bilayer of the endoplasmic reticulum of hepatocytes (Guengerich F. , 1992). P450s are organized into families based on amino acid homology and families are given a numerical designation—i.e. 1, 2, or 3. These families are further subdivided into subfamilies designated by capital letters—i.e. A, B, or C. Members within these subfamilies are delineated by numbers that represent an individual gene (Guengerich F. , 2010) (King, 2009) (Bibi, 2008) **(Figure 1.1).** Families 1, 2, and 3 are primarily involved in drug metabolism and metabolize over 70 % of clinical drugs (King, 2009).

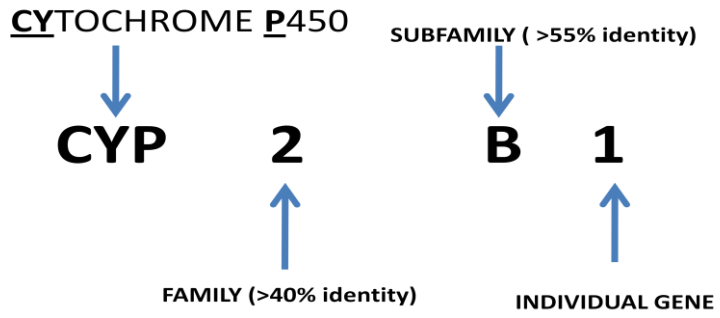


Figure 1.1: Nomenclature for Cytochrome P450s.

CYPs feature wide diversity in catalytic ability and can be induced by the same compounds that they metabolize. CYP inducers trigger the transcription of more CYP proteins. This ensures rapid detoxification of potentially harmful substances (Guengerich F. , 1991) (Guengerich F. , 2010). Understanding the induction of these microsomal enzymes via different compounds, and their interactions with one another, is important for developing novel therapeutics as well as predicting possible drug-drug interactions. The inductions of different CYPs such as CYP1A, CYP2B, and CYP3A are mediated by several ligand-activated transcription factors including the aryl hydrocarbon receptor (AHR), the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR), respectively.

1.2 Aryl Hydrocarbon Receptor (AHR)

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that regulates the transcription of the CYP1A and CYP1B subfamilies as well as other target genes. A member of the basic helix-loop-helix PER-ARNT-SIM transcription factory family (Yi-Zhong, Hogenesch, & Bradfield, 2000), AHR is primarily involved in mediating the response to environmental toxins such as 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) or the carcinogenic polycyclic aromatic hydrocarbons (PAHs) found in cigarette smoke (Li & Wang, 2010) (Abel & Haarmann-Stemmann, 2010). Besides its role in toxicology, the AHR is also known to play other important roles in development and the immune system. For example, AHR regulates Th17 cell differentiation (Kimura, Naka, Nohara, Fujii-Kuriyama, & Kishimoto, 2008); while studies with AHR knockout mice show that the AHR is involved in both neuronal

development (Gohlke, Stockton, Sieber, Foley, & Portier, 2009) and cardiovascular function (Zhang, 2011).

In its inactive state, the AHR is sequestered primarily in the cytoplasm, bound in a complex with heat shock protein (Hsp90), X-associated protein 2 (XAP2), and p23 (Petrulis & Perdew, 2002). Upon ligand binding, the AHR undergoes a conformational change and exposes a nuclear localization signal (Ikuta, Eguchi, Tachibana, Yoneda, & Kawajiri, 1998). The activated AHR then dissociates from this cytoplasmic complex and translocates to the nucleus where it dimerizes with its partner, AHR nuclear translocator (ARNT) (Reyes, Reisz-Porszasz, & Hankinson, 1992). In the nucleus, the AHR/ARNT heterodimer binds to xenobiotic response elements (XREs) located in the 5' flanking region of the target genes and then recruits the other components of the transcription initiation complex machinery.

These XREs contain an invariant 5'-GCGTG-3' core sequence recognized by AHR and ARNT heterodimer (Safe S. , 2001) . Alternatively, the AHR can also behave as a coactivator for the rat CYP1A2 gene where it associates with a binding factor that binds an enhancer sequence named XREII (Sogawa, et al., 2004). Finally, the AHR/ARNT dimerization and subsequent binding to the DNA leads to the *de novo* transcription of AHR target genes **(Figure 1.2a).**

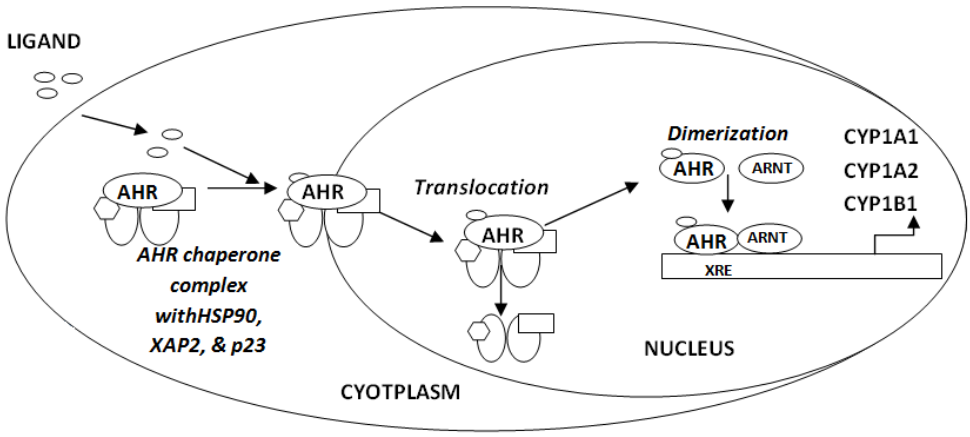


Figure 1.2: Mechanism of aryl hydrocarbon receptor activation and subsequent transcription of target genes.

The AHR can be activated by a wide range of structurally divergent ligands including polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs) and dioxins (Denison & Nagy, 2003). The AHR can bind such endogenous ligands such as the tryptophan metabolite kynurenic acid and exogenous ligands such as 3-methylcholanthrene (3MC) and beta-naphthoflavone (β-NF) **(Figure 1.2b).**

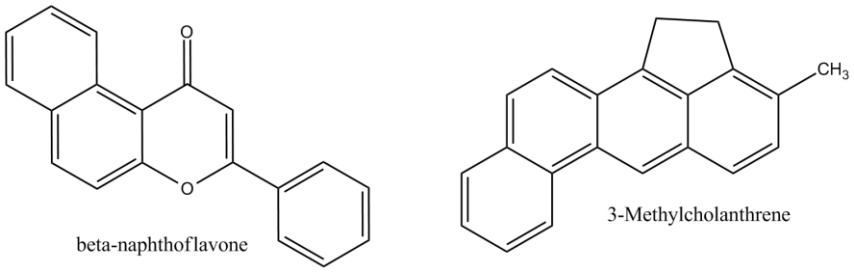


Figure 1.2b: Structures of AHR agonist beta-napthoflavone (β-NF) and 3-methylcholanthrene (3MC).

1.3 Constitutive Androstane Receptor (CAR)

First identified in 1994, orphan nuclear receptor CAR (NR1I3) is a xenobiotic and energy sensor that is abundantly expressed in the liver (Baes, Gulick, Choi, Martinoli, Simha, & Moore, 1994). Primarily involved in the regulation of enzymes that assist with xenobiotic and endobiotic metabolism, NR1I3 is constitutively active and can activate target genes without the presence of a ligand (Dussault, et al., 2002) (Xu, et al., 2004). This constitutive activity is suppressed by the binding of inverse agonists such as androstane metabolites and its activity is further activated by the presences of an agonist (Forman, et al., 1998). Thus, NR1I3 is known both as the constitutively active or the constitutive androstane receptor (CAR).

A member of the nuclear receptor superfamily, CAR is characterized both by a highly conserved zinc finger DNA binding domain (DBD) and by a ligand binding domain (LBD) that shows species differences in ligand specificity (Li & Wang, 2010)**.** CAR resides primarily in the cytoplasm bound to chaperone proteins such as the CAR retention protein (CCRP) and the heat shock protein (HSP90) (Kobayashi, Sueyoshi, Inoue, Moore, & Negishi, 2003). Upon activation, CAR dissociates from this

complex and translocates to the nucleus. In the nucleus, CAR dimerizes with its binding partner RXR (retinoid X receptor) and subsequently binds nuclear receptor sites containing DR4 repeats within the phenobarbital response element module (PBREM) of its target genes (Sueyoshi & Negishi, 2001) (Swales & Negishi, 2004). This translocation of CAR from the cytoplasm to the nucleus is triggered via two different mechanisms—either directly as in the case of the planar hydrocarbon 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) or indirectly as is the case with the antiepileptic phenobarbital (PB). The binding of TCPOBOP directly to CAR triggers the translocation of CAR and subsequent transactivation of its target genes (Tzameli, Pissios, Schuetz, & Moore, 2000) **(Figure 1.3a).** Activation of CAR by phenobarbital (PB) seems to be dependent on the dephosphorylation of a threonine 38 residue on CAR. If phosphorylated, CAR remains sequestered in the cytoplasm, and once dephosphorylated CAR translocates to the nucleus (Mutoh, et al., 2009). After treatment with okadaic acid, an inhibitor of serine/threonine phosphatases, the activation of CAR by PB was inhibited showing that the recruitment of a protein phosphatase 2A is necessary for CAR activation (Yoshinari, Kobayashi, Moore, Kawamoto, & Negishi, 2003) (Kawamoto T. , Sueyoshi, Zelko, Moore, Washburn, & Negishi, 1999). Other proposed mechanisms involve activation of AMP-activated protein kinase (AMPK) (Rencurel, et al., 2006). AMPK is an energy sensor and is activated when cells are energy depleted or when the AMP/ATP ratio is high. Rencurel and colleagues showed that AMPK activation stimulated CAR activation and CYP2B induction (Rencurel, et al., 2006), but it was later shown that AMPK activation alone is not sufficient for CYP2B induction (Shindo, Numazawa, & Yoshida, 2007). Recently, Osabe and Negishi reported that the phosphorylated (active) extracellular signal-regulated kinase (ERK) 1/2 represses CAR dephosphorylation keeping CAR sequestered in the cytoplasm (Osabe & Negishi, 2011). For a summary of phenobarbital's indirect activation of CAR see **Figure 1.3b.** Although the definitive mechanism for PB activation of CAR may not be clear, it is widely accepted that PB activation of CAR is dependent on a dephosphorylation mechanism. Overall, the activation of CAR regulates enzymes that are important for metabolism of

xenobiotics and fatty acid metabolism. Here, we are primarily interested in phenobarbital's ability to induce CYP2B via CAR activation.

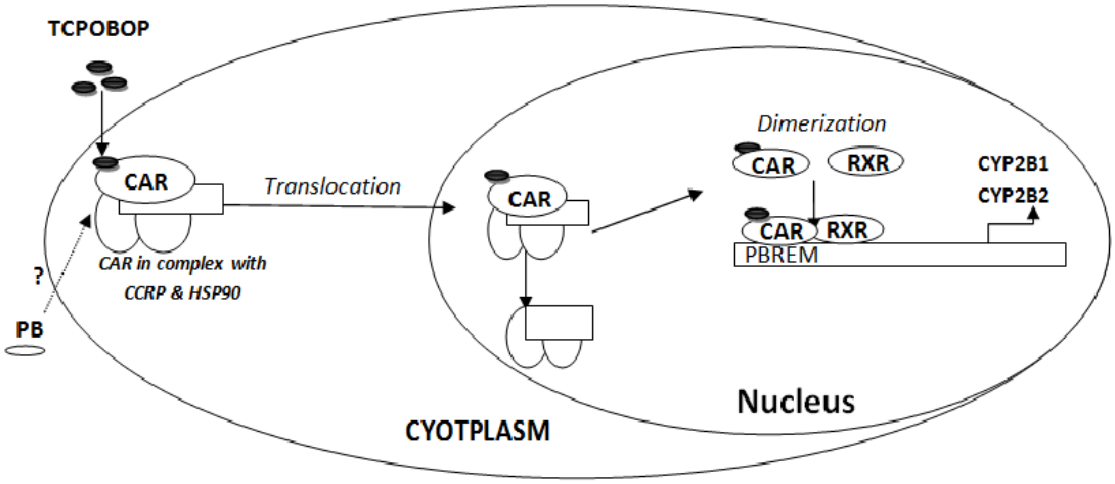


Figure 1.3a: Mechanism of constitutive androstane receptor (CAR) activation directly by TCPOBOP and indirectly by PB.

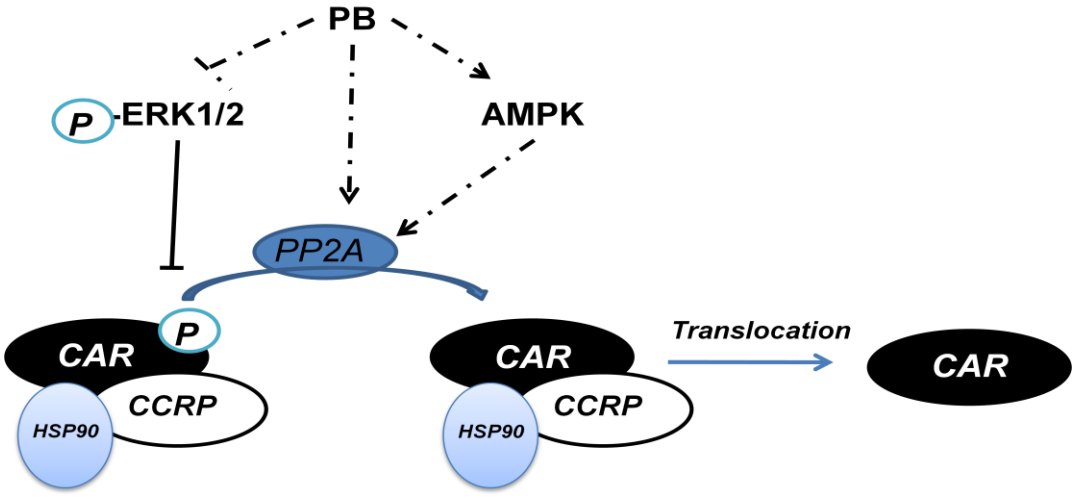


Figure 1.3b: Proposed mechanisms of indirect activation of CAR by phenobarbital (PB).

1.4 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR) or NR1l2 is a member of the nuclear receptor family that acts as a ligand-activated transcription factor (Kliewer S. , et al., 1998). Highly expressed in the liver, colon, and intestines PXR regulates genes primarily involved in metabolism and transport of drugs and bile acids (Kliewer S. A., et al., 1998) (Kliewer and Willson, 2002). Due to its large flexible binding pocket (Watkins R.E., et al., 2001) PXR can be activated by a wide variety of endogenous and exogenous compounds including steroids, bile acids, antibiotics, and herbal compounds (Kliewer, Goodwin, & Willson, 2002). PXR can also be activated by such ligands as pregnenolone 16α-carbonitrile (PCN), a rodent-specific agonist of PXR, as well as the synthetic glucorticoid steroid dexamethasone (DEX) **(Figure 1.4a)** (Jones, et al., 2000). Like CAR, PXR remains sequestered in the cytoplasm in a complex with CCRP and HSP90 (Squires, Sueyoshi, & Negishi, 2004). Upon ligand binding, PXR translocates to the nucleus where it forms a heterodimer with its binding partner retinoid X receptor (RXR) (Kliewer, SA, 2003). The binding of the PXR: RXR heterodimer to response elements triggers the subsequent transcription of PXR target genes—specifically CYP3As (Kliewer, SA, 2003) (Xie, et al., 2000).

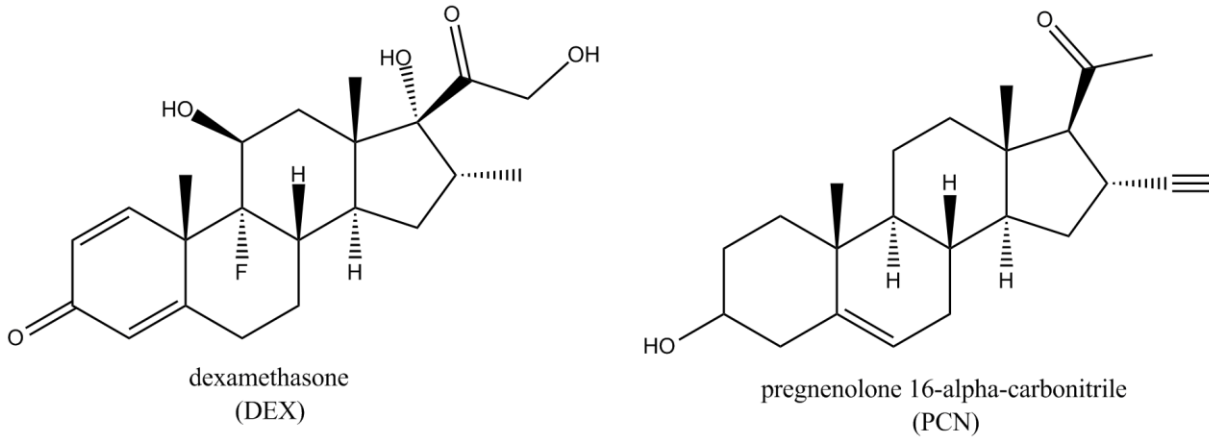


Figure 1.4a: Structure of PXR agonists' dexamethasone (DEX) and pregnenolone 16-alpha-carbonitrile (PCN).

The nuclear receptors CAR and PXR are known to directly regulate the transcription of CYP2B and CYP3A, respectively. However, there is some overlap between these two receptors (Wei, Zhang, Dowhan, Han, & Moore, 2002). Partial overlap exists with both receptors being able to bind the some of the same compounds with different affinities (Moore, et al., 2000) and more overlap exists when CAR and PXR bind to each other's response elements (Wei, Zhang, Dowhan, Han, & Moore, 2002). CAR has the ability to bind to PXR response elements in CYP3A genes while PXR can bind to PBREM in CYP2B genes. Although, PXR can bind to binding sites within the PBREM, the PBREM shows preference for the CAR/RXR heterodimer. (Wie, et al., 2000) (Xie, et al., 2004) (**Figure 1.4b**). Finally, this overlap between PXR and CAR has implications for possible drug-drug interactions.

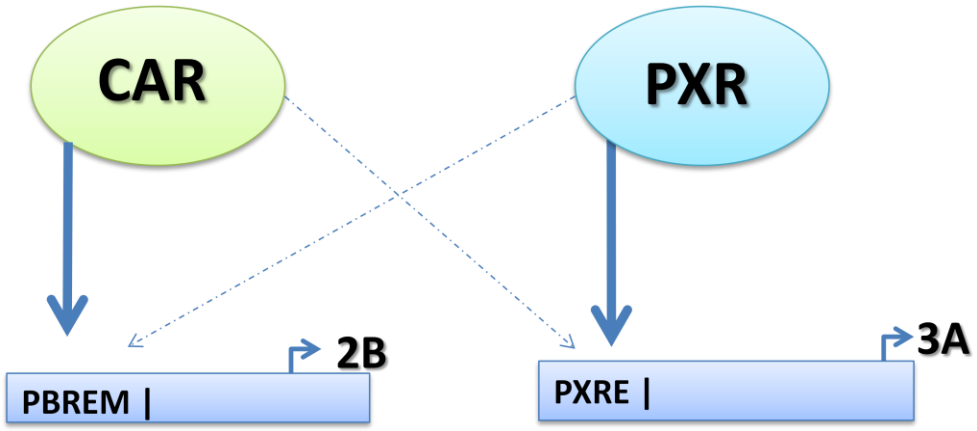


Figure 1.4b: Overlap between CAR and PXR.

1.5 Drug-Drug Interactions

Drug-drug interactions (DDIs) occur when the administration of one drug modifies the effect of another drug. This can be the consequence of pharmacodynamic changes at the site of action that lead to synergistic, additive, or antagonistic effects, or it can be due to pharmacokinetic changes or alterations in the absorption, excretion, and metabolism of a drug (Rang, Dale, Ritter, & Gardner, 1995). A drug that causes the alteration of another drug's action is called the perpetrator while the drug whose effects become altered is the victim (Prakash & Vaz, 2009). DDIs frequently lead to a loss of therapeutic efficacy, potential toxic effects, or increased bioavailability. Identifying potential DDIs has huge clinical

implication with the use of multi-drug therapies as well as patients who take multiple medications for multiple conditions. Diet as well as herbal remedies can also change the action of drugs. For example, grapefruit has been known to inhibit CYP3A4 and change the bioavailability of drugs such as the calcium blocker felodipine (Bibi 2008) (Bailey, Malcolm, Arnold, & Spence, 1998). Identifying possible drug-drug interaction *in vitro* is an important step in drug development that will help to prevent potentially adverse effects.

DDIs more than often result from changes in metabolism. Specifically, the induction or inhibition of phase I metabolizing enzymes by one drug can change the disposition of another drug (Deshmukh, 2009) (Prakash & Vaz, 2009). Because nuclear factors (CAR, PXR, AHR) mediate the transcription of drug metabolizing enzymes and are also activated by the same compounds these enzymes metabolize, identifying any possible overlap or cross-talk between NRs can give insight for potential DDIs. So far there has been evidence of cross-talk between CAR and PXR (Wei, Zhang, Dowhan, Han, & Moore, 2002), PXR and other NRs (Kumar, Jaiswal, Kumar, Negi, & Tyagi, 2010), and AHR and the estrogen receptor (ER) (Safe & Wormke, 2003) (Ohtake, et al., 2003). However, at present there have been no definitive reports of cross-talk between the AHR and CAR.

1.6 Proposal

A previous finding in our lab demonstrated that CYP2B1 protein induction was impaired after treating primary rat hepatocytes with a combination of known CAR, AHR, PXR, and peroxisome proliferator-activated receptor alpha (PPAR) ligands (Dr. Choon Lee unpublished data). After further investigation, Dr. Lee discovered that somehow the addition of β-NF, an AHR agonist, in combination with PB inhibited PB-dependent induction of CYP2B. This study was started in an effort to further establish this phenomenon and to begin to elucidate if and how β-NF via AHR repressed the induction of CYP2B. We have used combinations of AHR, CAR, and PXR ligands in primary rat hepatocytes to determine if this

phenomenon, i.e. antagonism of PB-dependent induction of CYP2B is CAR: AHR mediated or PXR: AHR mediated. Real-time *q*PCR as well as Western blot helped us to observe changes in gene expression and subsequently activation of these nuclear receptors. Furthermore, we showed that the repression of PB-dependent induction of CYP2B with AHR agonist happens at the transcriptional level. We also began mechanistic studies looking at the translocation of CAR as well as attempts to inhibit AHR via AHR antagonists. Finally, we will speculate how AHR activation inhibits PB-dependent induction of CYP2B1.

2. Materials and Methods

2.1 Materials and Reagents

Chemicals used for treatments: β-NF, PB, TCPOBOP, 3MC, KA, PCN, dexamethasone (water soluble) were all acquired from Sigma-Aldrich (St. Louis, MO) as well as Williams' E medium, Krebs-Ringer buffer, collagenase and other general chemicals.

Antibodies to CYP2B1 and CYP3A2 were provided by Dr. James Halpert (University of California, San Diego, CA), and anti-CYP1A antibody was purchased from Daichi Pure Chemicals (Tokyo, Japan). The anti-GAPDH monoclonal antibody was acquired from Millipore (Billereca, MA) and the anti-CAR polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Preparation of Rat Hepatocytes and Cell Culture

Primary rat hepatocytes were isolated by a two step in situ collagenase perfusion procedure as previously described (Sewer & Morgan, 1997). Briefly, male F344 rats (180-250g) were anesthetized using a ketamine-xylazine solution and the liver was perfused via the portal vein with Krebs-Ringer-bicarbonate buffer followed by media containing 0.3 mg/ml collagenase. The liver was harvested and then mechanically separated to release cells. The cells were placed on collagen coated plates with plating media containing 10 % FBS (Atlanta Biologicals, Norcross, GA) in complete Williams E media. Four hours later the cells were overlaid with media containing Matrigel (0.234 mg/ml, BD biosciences, San Jose, CA). The media was changed to serum-free Williams E containing 10 mM HEPES pH 7.4, 10 nM insulin, 25 nM dexamethasone and 10 mg/ml penicillin/streptomycin after 24 hours. Cells were incubated in 5% CO_2 at 37 ^{o}C until harvest. All treatments were made 72 hours after initial plating.

2.3 RNA Extraction and Reverse Transcriptase Real-time qPCR

RNA was extracted from 12-well plates using RNA-Bee reagent (Tel-Test; Friendswood, TX) according to the manufacture's protocol. RNA concentration was estimated using 260/280 nm spectrometric method and cDNA was synthesized by using the high capacity cDNA Archive kit from Applied Biosystems (Foster City, CA). The reaction was then incubated at 25 °C for five minutes, 37 °C for two hours, 85 °C for five seconds, and then held at 4 °C. Real-time PCR was carried out using SYBR Green PCR Master mix (Applied Biosystems, Foster City CA) and the ABI prism 7300 real-time PCR system. Primer sequences (Table 1) were obtained from previously published papers and can be found in the table below. Analysis of real-time PCR was carried out via the ΔΔCt method as described by Livak and Schmittgen (Livak et al., 2001) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as the normalization control.

Table 1. Primer sequences used.

Gene	**Primer Sequence**	**Reference**
CYP2B1 & CYP2B2	2B1antisense:TCACACCGGCTACCAACCCT 2B2 antisense: TCTCACAGGCACCATCCCT 2B1/2B2 sense: CTGTGGGTCATGGAGAGCTG	(Li & Kupfer, 1998)
CYP3A2	Sense: TACTACAAGGGCTTAGGGAG Antisense: CTTGCCTGTCTCCGCCTCTT	(Hoen, 2000)
CYP1A1	Sense: GGGTGTTGAGAGGCACAAGG Antisense: CACTAGGGCCTGCTTGATGG	(Elbarbry, 2007)
GAPDH	Sense: TGCCAAGTATGATGACATCAAGAAG Antisense: AGCCCAGGATGCCCTTTAGT	(Lee, Kim, Lian, & Morgan, 2008)

2.4 Protein Extraction and Immunoblotting

After the addition of cold cell lysis buffer (50 mM Tris, pH 7.5, 0.1 % SDS, 0.5 % Nonidet P-40, 1 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich, St. Louise, MO), cells were scraped and collected. For cell lysates, the cells were sonicated for 7-10 seconds at power 15 using a Microsan ultrasonic cell disruptor Microsan (Misonix Inc., Farmingdale, NY). Then, the cells were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was then used for SDS-polyacrylamide gel electrophoresis (PAGE). The quantity of protein was analyzed by using a Pierce BCA protein assay kit (Rockford, IL) according to the manufacture's protocol. Equal amounts of protein were loaded on the gel. After electrophoresis, blots were blocked in 3 % nonfat dry milk in washing buffer (10 mM potassium phosphate buffer, pH 7.5, 0.05 % Tween, 0.01 % NaN_3, 1.15 % KCl) for 1 hr at room temperature. The blots were probed overnight with the following antibodies at 4 °C: Anti-CYP2B1 antibody (diluted 1:10,000), anti-1A antibody (diluted 1:5,000), anti-CYP3A2 antibody (diluted 1:5,000), anti-GAPDH (diluted 1:10,000), and anti-CAR antibody (diluted 1:200). Following a brief wash, secondary antibodies were added for 1 hr at room temperature. Chemiluminescence was detected with enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL) on X-ray film and the intensity of the bands was estimated by using Gel-Doc (Bio-Rad, CA) with Image Lab software (ver. 4.0, Bio-Rad).

2.5 Preparation of Nuclear and Cytosolic Fractions for CAR blotting

The following protocol was adapted from (Li, Lii, Yang, & Chen, 2007). After harvest cells were placed in a -20 °C freezer and 0.25 mL of cell lysis buffer (50 mM Tris, pH 7.5, 0.1 % SDS, 0.5 % Nonidet P-40, 1 mM EDTA, protease inhibitor mixture; Sigma-Aldrich) was added to each well. Cells were collected with a cell scraper and the samples were briefly sonicated for five seconds at power 3. The samples were allowed to incubate on ice for 10 min while vortexing briefly and then centrifuged at 700*g* for five minutes at 4 °C. The supernatant was collected for the cytosolic fraction and the crude pellet was use for the nuclear

fraction. To purify the nuclear fraction, 40 µL of buffer B (146 mM sucrose, 100 mM KCl, 5 mM MgCl2, 0.5 mM CaCl2, 10 mM Tris-HCL 7.5 pH, and 0.1 mM PMSF) was added to each sample. The samples were shaken for 30 minutes at 4 °C and then centrifuged at 1600 g for 10 min at 4 °C. The supernatant was removed and collected. The pellet was then rewashed with 50 µL of buffer B, shaken for 30 min at 4 °C, and centrifuged at 1600*g* for 10 min at 4 °C. The remaining supernatant was added to the previously collected supernatant and the total volume (90 µL) was used for the nuclear protein. For the cytosolic fraction, the samples were sonicated for seven seconds at power 15 and then centrifuged at 12000*g* for 10 min. The final supernatant was used for the cytosolic fraction and the amount of protein in each sample was analyzed using the BCA assay as previously described. Levels of CAR protein expression were observed using SDS-PAGE and western blotting as previously described.

2.6 Statistical Analysis

Differences between treatments were calculated using one-way analysis of variance (ANOVA) followed by a Tukey test, Games-Howell, or Dunnett's test as appropriate. Significance was set at $p<0.05$.

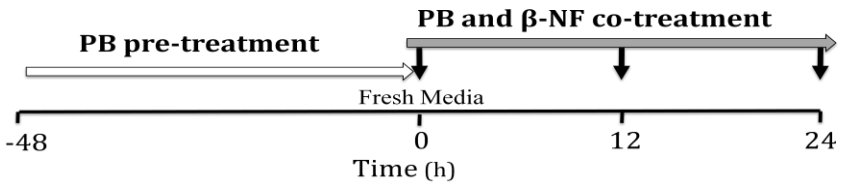
3. Results

3.1 Effect of β-NF on CYP2B1 RNA levels after pretreatment with PB

Beta-naphtoflavone (β-NF) is a prototypical activator of AHR and has been used in several studies exploring AHR transactivation and subsequent CYP1A induction (Timme-Laragy, Cockman, Matson, & Di Giulio, 2007) (Stegeman, et al., 1995). To observe the effects of co-treatment with β-NF and phenobarbital (PB), a prototypic CAR agonist (Kawamoto T. , Sueyoshi, Zelko, Moore, Washburn, & Negishi, 1999), primary rat hepatocytes were pre-treated for 48 hrs with 1 mM PB. Following the pre-treatment, cells were then treated with 1 mM PB with or without 20 µM of β-NF for an additional 12 or 24 hours and then harvested (**Schematic 3.1**). Phenobarbital maintained CYP2B1 expression as expected in a time-dependent manner (**Figure 3.1**). However, concomitant addition of β-NF down-regulated the CAR dependent expression of CYP2B1 at the 12 and 24 hr time points with a significant decrease in CYP2B1 expression at the 24 hr time point when comparing PB and β-NF co-treatment to PB alone.

3.2 Effect of CAR agonists with or without β-NF on CYP2B and CYP1A RNA

As stated earlier, CAR can be activated via two mechanisms: indirectly as in the case with PB, and directly as in the case with TCPOBOP. Since the preceding results suggested that β-NF acting through AHR attenuated PB-dependent activation of CAR, we wanted to determine if this attenuation of CYP2B mRNA expression was a PB-dependent phenomenon. Additionally, unlike the previous experiment cells were not pre-treated with PB before co-treatment with β-NF. This was done in order to see if the same attenuation of CYP2B expression would occur with simultaneous co-treatments versus the staggered co-treatment of the previous experiment. Primary rat hepatocytes were treated with a combination of TCPOBOP or PB with β-NF for 12, 24, and 48 hours (**Schematic 3.2**).



Scheme 3.1: Experimental design to observe the effect of β-NF on CYP2B1 mRNA levels after pretreatment with PB. Cells were treated with a CAR activator, PB, in or out of the presence of 20 µM β-NF after 48 hour pretreatment with 1 mM PB. Cells were then harvested 12 and 24 hours later and the CYP2B1 mRNA levels were observed.

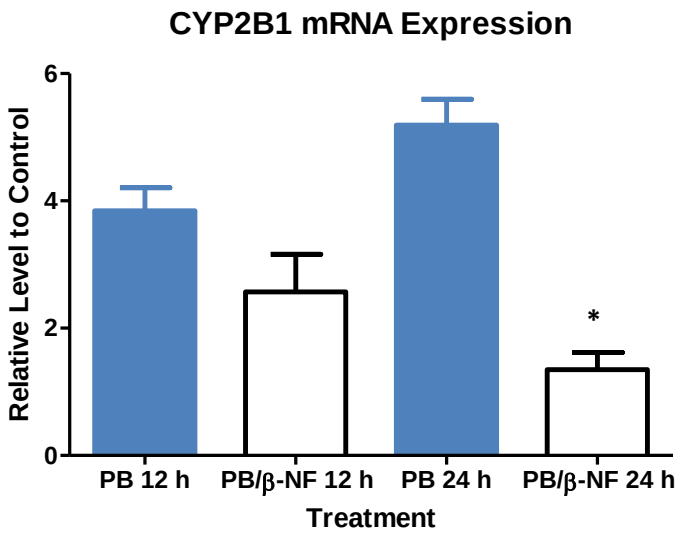


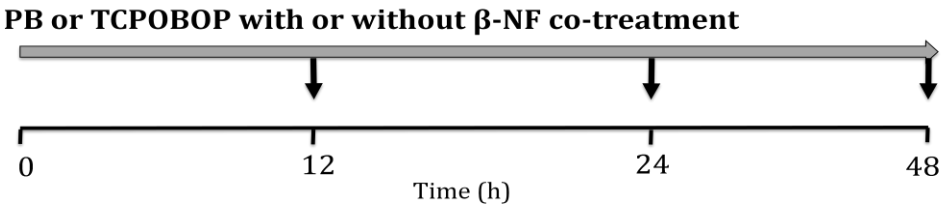
Figure 3.1: Effect of PB and β-NF co-treatment after 48 hr pretreatment with PB on CYP2B mRNA levels. Primary rat hepatocytes were treated with 1 mM PB with or without 20 µM β-NF after 48 hr exposure to PB alone. PB alone increased CYP2B expression in a time-dependent manner while β-NF co-treatment down-regulated CYP2B1 expression. Symbol (*) indicates significance to PB 24 h where $p<0.05$.

For CYP2B1 and CYP2B2, PB strongly increased CYP2B1 and CYP2B mRNA expression at all time points (**Figure 3.2a and Figure 3.2b**). β-NF, an AHR agonist, transiently increased CYP2B1 and CYP2B2 expression at the 12 hr time-point. β-NF is known to increase CYP1A expression, but not CYP2B expression (Shimamoto, et al., 2011). When comparing the PB and β-NF co-treatment to PB alone at comparable time-points, the β-NF co-treatment once again attenuates CYP2B expression relative to PB alone. This attenuation of CYP2B expression is significant at the 48 h time point for CYP2B1 and at the 24 h time point for CYP2B2 (**Figure 3.2a and Figure 3.2b**). TCPOBOP also increased CYP2B1 and CYP2B2 expression but not as efficaciously as PB. Co-treatments with TCPBOBOP and β-NF attenuated CYP2B1 and CYP2B2 expression at the 24 and 48 h time points with a significant decrease at the 24 h time point for CYP2B1 (**Figure 3.2a and Figure 3.2b**).

For CYP1A2, β-NF alone robustly increased mRNA levels in a time-dependent manner. Additionally, all treatments that contained β-NF (β-NF alone, TCPOBOP/β-NF, and PB/β-NF) also increased CYP1A2 expression in a time-dependent manner. Because the CAR agonists by themselves did not significantly increase CYP1A2 expression, this increase in CYP1A2 expression with co-treatments is more than likely due to the β-NF effect alone (**Figure 3.2c**).

3.3.1 Concentration dependence of Exogenous AHR agonist β-NF's Effects on CYP2B1 and CYP1A2 Gene Expression

For the following experiments, primary rat hepatocytes were treated with increasing concentrations of an AHR agonist with or without the presence of 1 mM PB. This was done in order to determine the concentration of the AHR agonist that would best attenuate CYP2B gene expression. Cells were harvested after 24 hours for CYP2B1 mRNA levels and in a subsequent experiment, protein expression was determined after 48 hours of exposure (**Scheme 3.3**).



Scheme 3.2: Experimental Design for simultaneous co-treatment with CAR activators with or without β-NF. Cells were simultaneously treated with a CAR activator, PB or TCPOBOP, with or without the presence of 20 µM β-NF. Cells were then harvested 12, 24, and 48 hours after the initial treatment and CYP2B mRNA expression was observed.

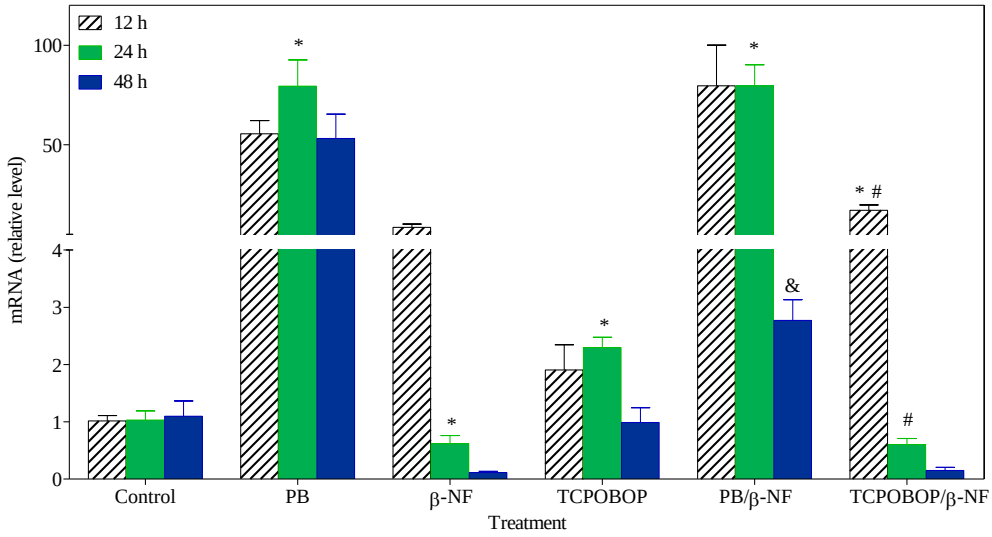


Figure 3.2a: Effect of simultaneous treatment with β-NF on CYP2B1 induction by CAR activators. Cells were harvested 12, 24, and 48 h after treatment and CYP2B1 mRNA levels were determined by Real-Time RT-PCR. Symbols indicate significant difference ($p<0.05$) from control (*), TCPOBOP (#) and PB (&).

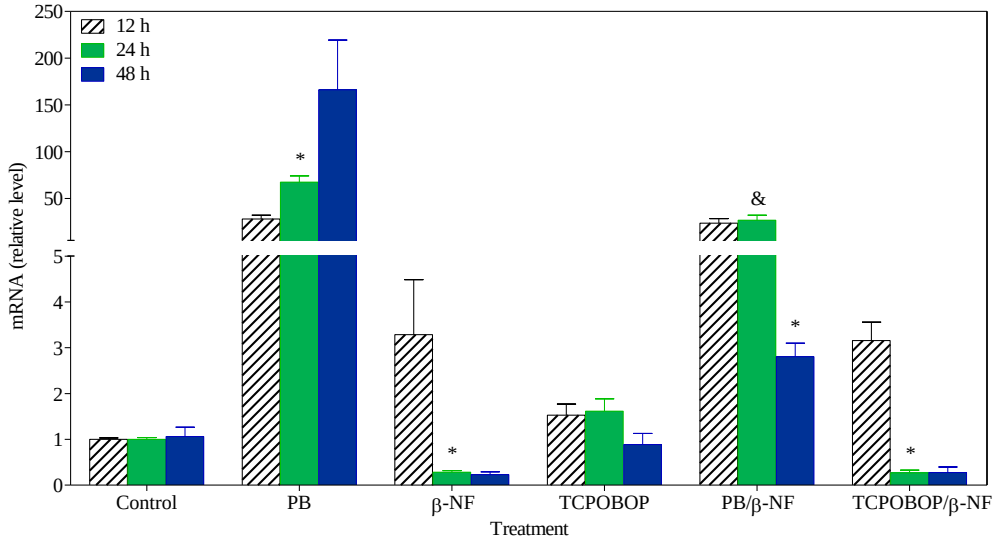


Figure 3.2b: Effect of simultaneous treatment with β -NF on CYP2B2 induction by CAR activators. Cells were harvested 12, 24, and 48 h after treatment and CYP2B2 mRNA levels were determined by Real-Time RT-PCR. Symbols indicate significant difference ($p<0.05$) from control (*) and PB (&).

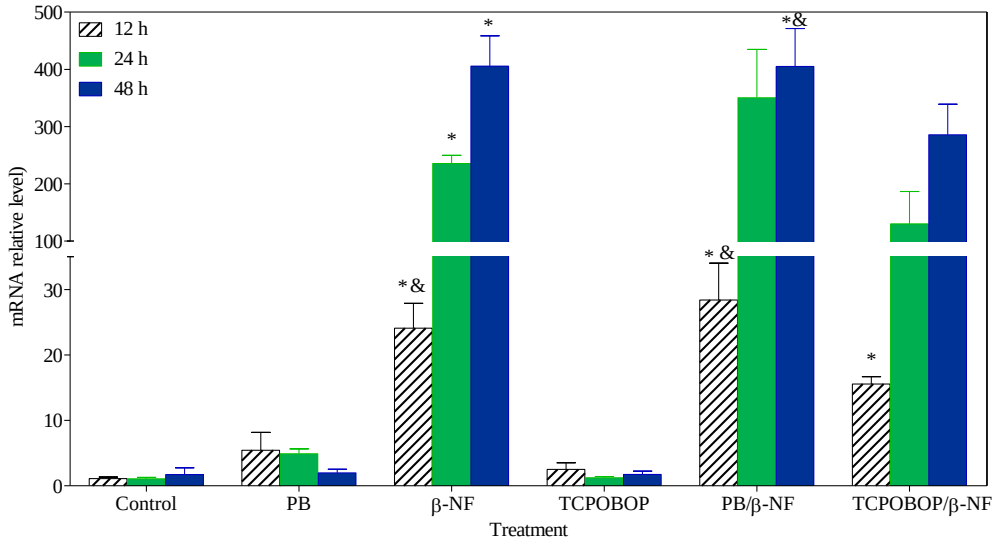


Figure 3.2c: Effect of simultaneous treatment with β-NF on CYP1A2 induction by CAR activators. Cells were harvested 12, 24, and 48 h after treatment and CYP2B2 mRNA levels were determined by Real-Time RT-PCR. Symbols indicate significant difference ($p<0.05$) from control (*) and PB (&).

β-NF alone increased CYP1A2 expression in a dose-dependent manner as expected (Traber, McDonnel, Wang, & Florence, 1992) with a half-maximal induction of CYP1A2 occurring between 1 µM and 10 µM. Significant induction of CYP1A2 in relationship to the control occurred at the 1 µM, 10 M, 40 µM, and 100 µM concentrations of β-NF (**Figure 3.3a**).

CYP2B1 expression was significantly attenuated at 10 µM concentration of β-NF with 1 mM PB co-treatment (**Figure 3.3b**). This suggests that with co-treatment with 1 mM PB, a 10 µM β-NF dose is the most effective at attenuating CYP2B1 expression. Finally, the potency of CYP2B1 down-regulation by β-NF is similar to the potency of β-NF on CYP1A2 induction

After 48 hours of exposure to 1 mM PB and 10 µM β-NF, CYP2B protein levels were attenuated by PB and β-NF co-treatment compared to PB alone (**Figure 3.3c**). Additionally, PB alone increased significantly CYP2B expression when compared to control as expected. Interestingly, β-NF treatment alone decreased CYP2B protein levels when compared to control (**Figure 3.3c**). Finally, CYP1A2 protein levels were also observed after 48 hrs and only the treatments that contain β-NF had an increase in CYP1A2 expression (data not shown).

Scheme 3.3: AHR agonists were treated with or without 1 mM PB to see their relative potencies. RNA was harvested after 24 h and protein was harvested after 48 hrs of treatment.

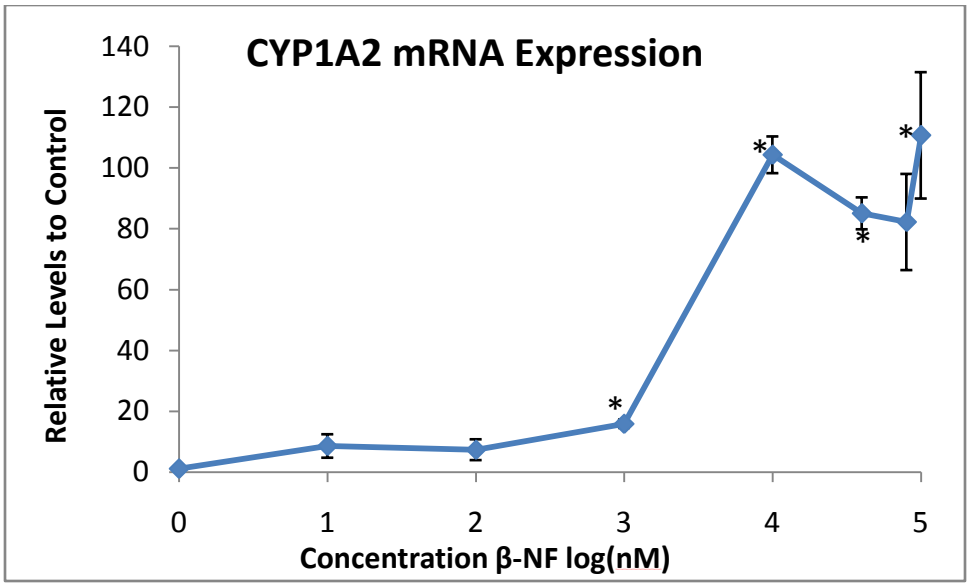


Figure 3.3a: Dose-dependent Response of β-NF on CYP1A2 mRNA expression. Primary rat hepatocytes were exposed to increasing concentrations of β -NF for 24 hours and CYP1A2 levels were determined via Real Time RT-PCR. Symbol (*) indicates significance from control ($p<0.05$).

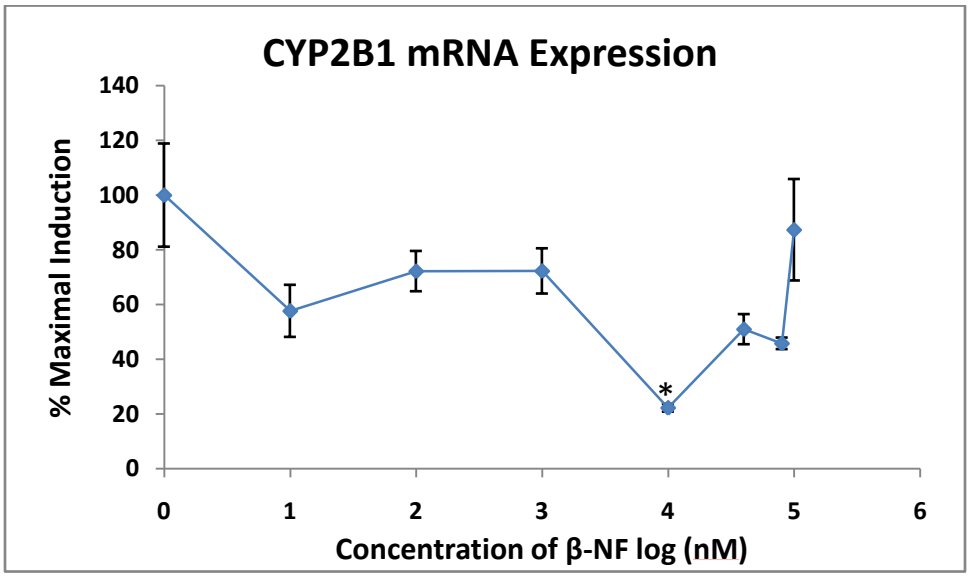


Figure 3.3b: Effect of concomitant treatment with 1 mm PB and increasing concentrations of β-NF on CYP2B1 mRNA expression. Cells were co-treated for 24 hours after which CYP2B1 levels were determined via real time RT-PCR. Symbol (*) indicates significance to 1 mM PB alone or maximal induction of CYP2B1.

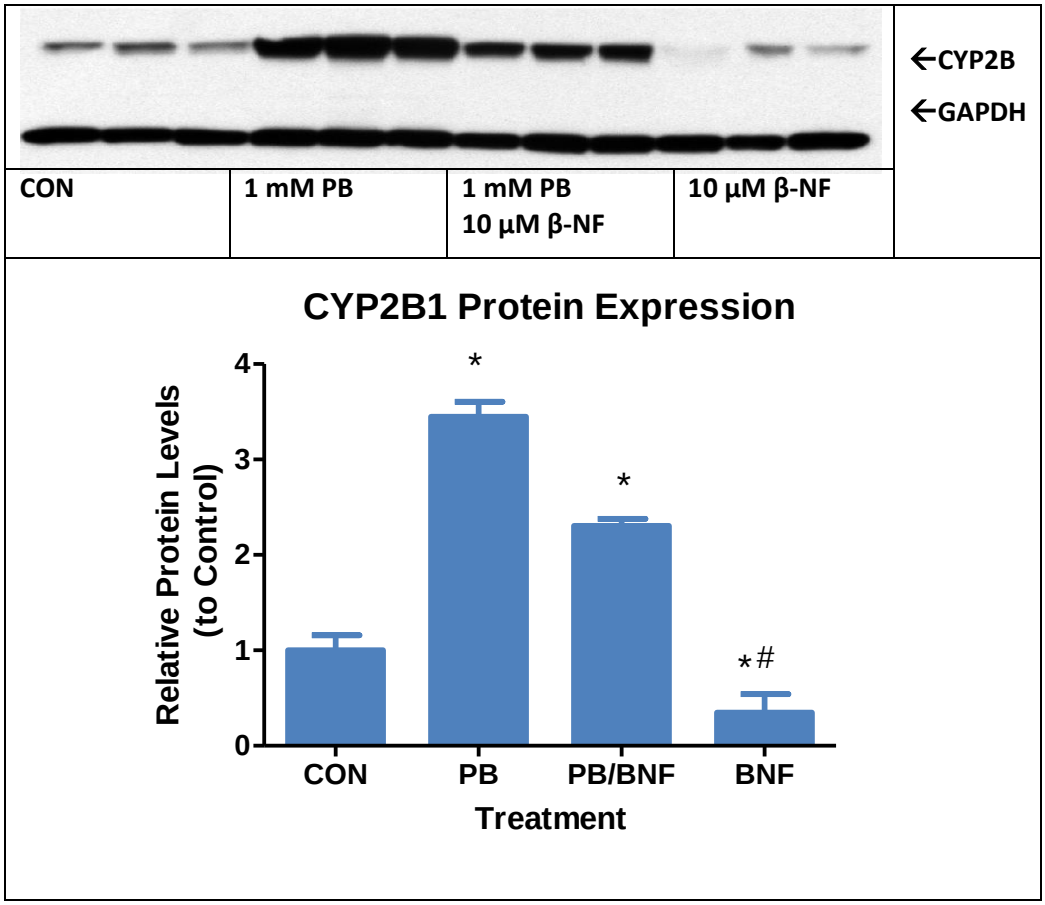


Figure 3.3c: Effect of PB and β-NF co-treatment on CYP2B protein expression. Primary rat hepatocytes were co-treated with PB and β-NF for 48 hours after which CYP2B protein levels were observed via Western blot. Symbols * and # indicate significance from control and PB alone, respectively ($p<0.05$).

3.3.2 Effect of the Exogenous AHR ligand 3MC on CYP2B and CYP1A Gene Expression

3-methylcholanthrene (3MC) is a potent activator of AHR and has been shown to significantly induce CYP1A levels (Masubuchi & Okazaki, 1997). Primarily used for the formation of murine sarcomas (Eisen, 1946) (Mider & Morton, 1939), 3MC is a planar polycyclic aromatic hydrocarbon typical of many AHR ligands (**Figure 1.2b**). Here we use 3MC, to see if a structurally different AHR activator would have the same effect on PB-dependent induction of CYP2B. Primary rat hepatocytes were treated with an increasing concentration of 3MC (10 nM-100 μM) with or without the presence of 1 mM PB. RNA was harvested after 24 hrs and in a subsequent experiment protein was harvested after 48 hrs of exposure with a combination of 10 μM 3MC and 1 mM PB. 3MC increased CYP1A2 mRNA expression in a dose-dependent manner with an approximate half maximal induction between 10 μM and 100 μM. Additionally, there was a significant increase in CYP1A2 mRNA expression at the 1μM, 10 μM, and 100 μM concentrations (**Figure 3.3d**). Interestingly, the fold increase in CYP1A2 caused by 3MC was 10 times greater than by β-NF.

For CYP2B, co-treatment with 3MC and PB decreased CYP2B1 mRNA expression at the 10 μM and 100 μM concentration (**Figure 3.3e**). After 48 hours of exposure to 1 mM PB and 10 μM 3MC, CYP2B protein levels were significantly attenuated with PB and 3MC co-treatment compared to PB alone. Additionally, PB alone significantly increased CYP2B expression when compared to control as expected while 3MC alone decreased CYP2B expression when compared to the control (**Figure 3.3.f**). CYP1A2 protein levels were also observed after 48 hrs and only the treatments that contain β-NF had an increase in CYP1A2 expression (data not shown). Finally, when comparing the potency of β-NF versus 3MC, 3MC is a less potent inducer of CYP1A2 than β-NF. However, 3MC is more efficacious than β-NF. Both compounds, although structurally different, attenuate the PB-dependent induction of CYP2B at the 10 μM concentration.

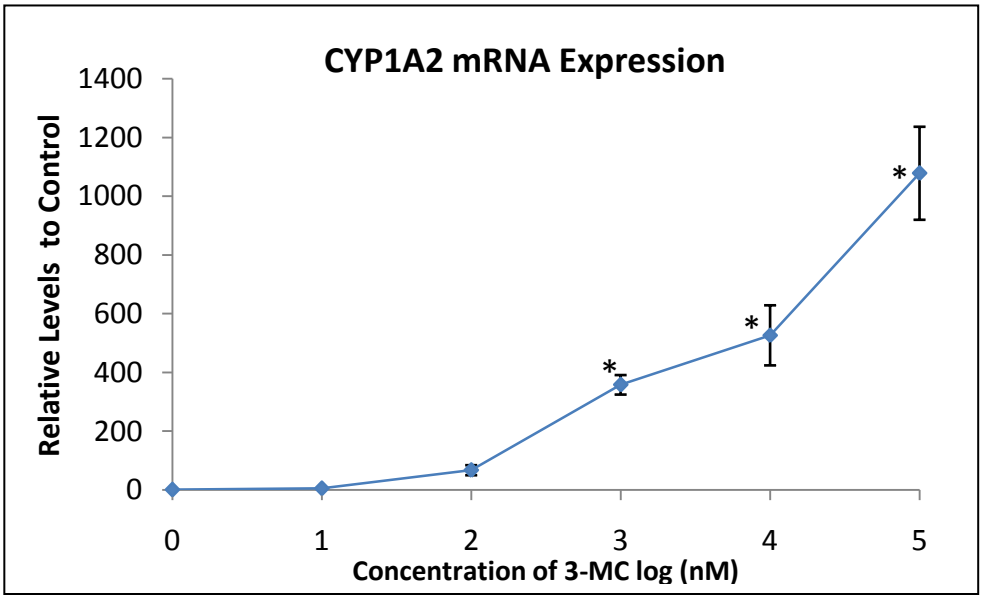


Figure 3.3d: Dose-dependent response of 3MC on CYP1A2 mRNA expression. Primary rat hepatocytes were treated with increasing concentrations of 3MC (10 nM -100 µM) for 24 hours after which CYP1A2 levels were observed via real-time RT-PCR. Symbol (*) indicates significance from control ($p<0.05$).

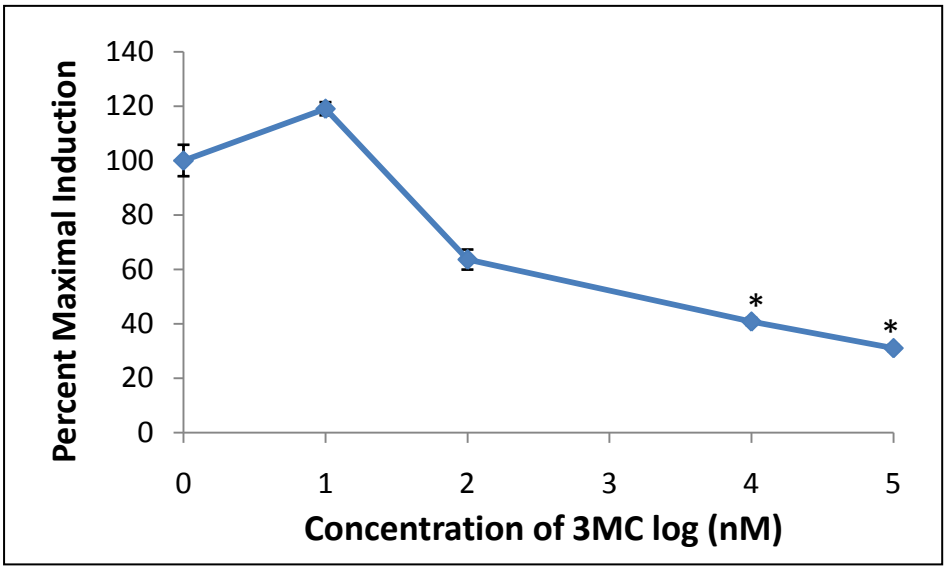


Figure 3.3e: Effect of concomitant treatment with 1 mm PB and increasing concentrations of 3MC on CYP2B1 mRNA expression. Cells were co-treated for 24 hours after which CYP2B1 levels were determined via real time RT-PCR. Symbol (*) indicates significance to 1 mM PB alone. Note concentration point 1 µM was removed because of abnormal GAPDH levels.

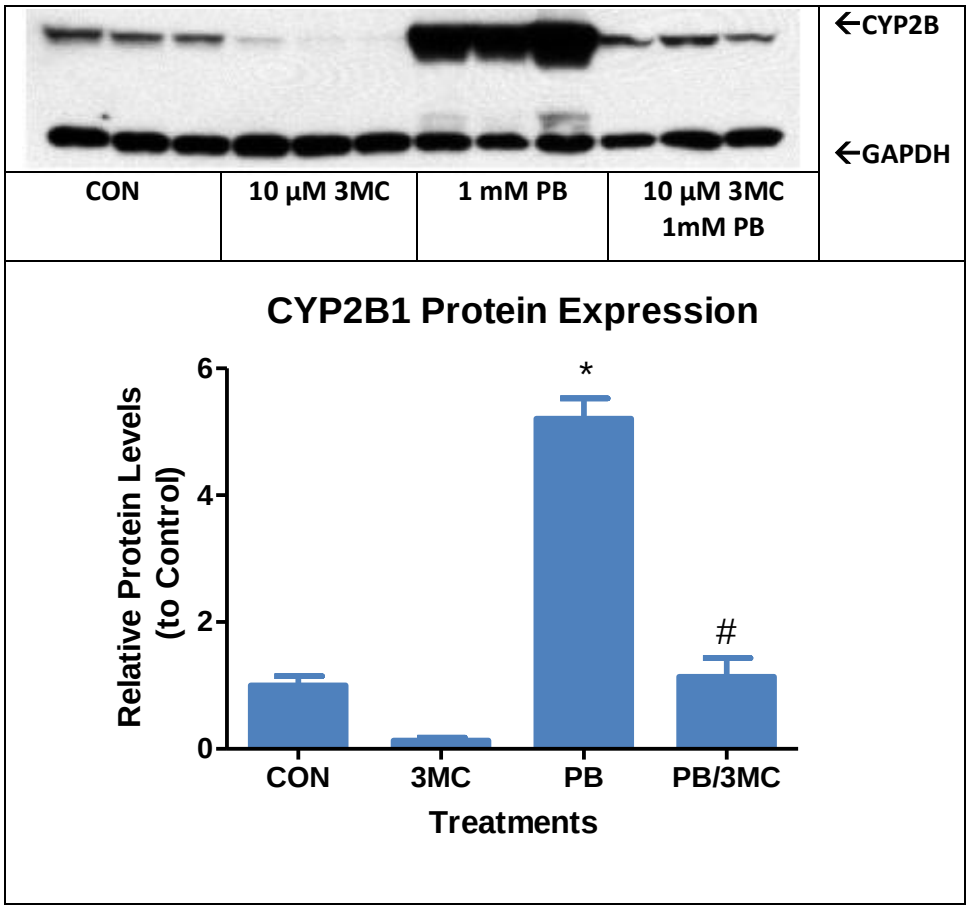


Figure 3.3f: Effect of PB and 3MC co-treatment on CYP2B protein expression. Primary rat hepatocytes were co-treated with PB and 3MC for 48 hours after which CYP2B protein levels were observed via Western blot. Symbols (*) and (#) indicate significance from control and PB alone, respectively ($p<0.05$).

3.3.3 Effect of the Endogenous AHR ligand KA on CY2B and CYP1A Gene Expression

Tryptophan metabolites and derivatives have been shown to activate the AHR (Heath-Pagliuso, et al., 1998) (Nguyen & Bradfield, 2008). A recent study by DiNatale and colleagues reported kynurenic acid (KA), a product of tryptophan oxidation, as a potent endogenous ligand for human AHR (2010). Kynurenic acid was shown to increase CYP1A mRNA levels in HepG2 hepatoma cells as well as being capable of binding AHR in competition binding assays (DiNatale B. , et al., 2010). In an attempt to see if an endogenous ligand of AHR would have the same repressive effects on CYP2B1 expression, as in the case with 3MC and β-NF, hepatocytes were co-treated with varying concentration of KA with or without 1 mM PB.

Kynurenic acid did not significantly increase CYP1A2 expression until the 100 µM concentration (**Figure 3.3g**). Increasing the concentration of KA above 1 mM was not done due to concern with cell viability. In a subsequent experiment with varying concentrations of KA (10 nM-1mM) and 1 mM PB, KA attenuated CYP2B1 mRNA expression at the 1 mM concentration. (**Figure 3.3h**).

After 48 hours of exposure to 1 mM PB and 100 µM KA, CYP2B protein levels were probed. Canonically, PB alone dramatically increased CYP2B expression while with co-treatment with PB and KA there was not a significant decrease in CYP2B protein expression. KA alone did not induce CYP2B nor did it decrease CYP2B when compared to control (**Figure 3.3i**).

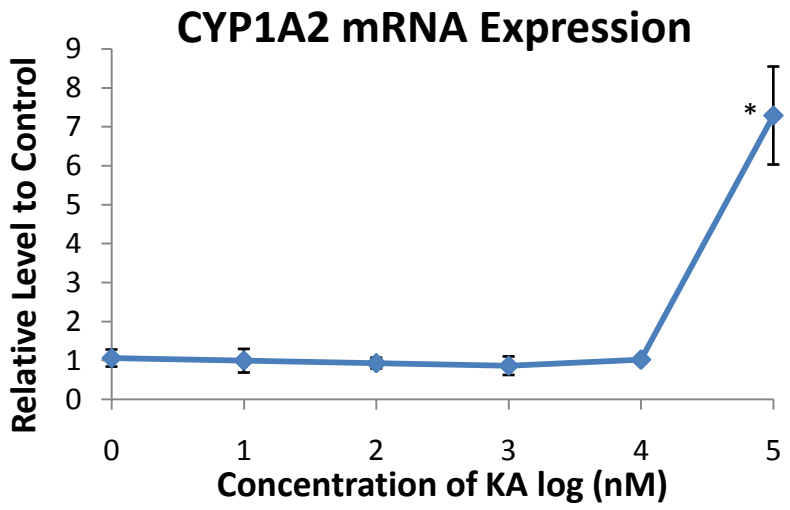


Figure 3.3g: Dose-dependent response of KA on CYP1A2 mRNA expression. Primary rat hepatocytes were treated with increasing concentrations of KA (10 nM -100 µM) for 24 hours after which CYP1A2 levels were observed via real-time RT-PCR. Symbol (*) indicates significance from control ($p<0.05$).

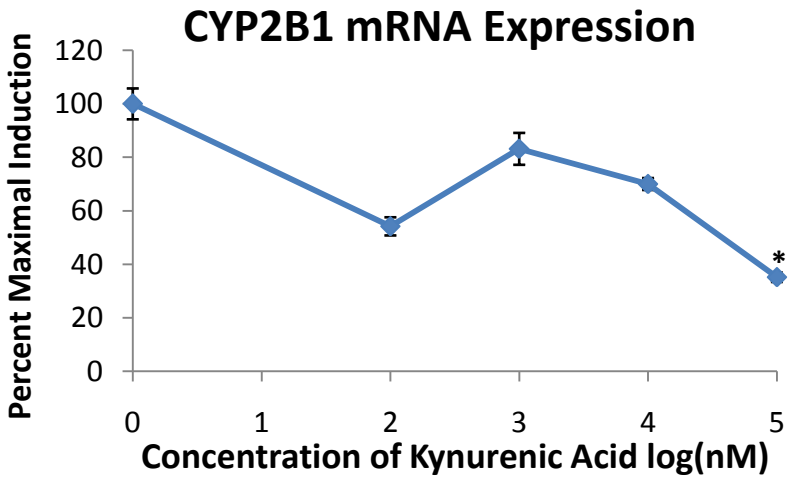


Figure 3.3h: Effect of concomitant treatment with 1 mm PB and increasing concentrations of KA on CYP2B1 mRNA expression. Cells were co-treated for 24 hours after which CYP2B1 levels were determined via real time RT-PCR. Symbol (*) indicates significance to 1 mM PB alone.

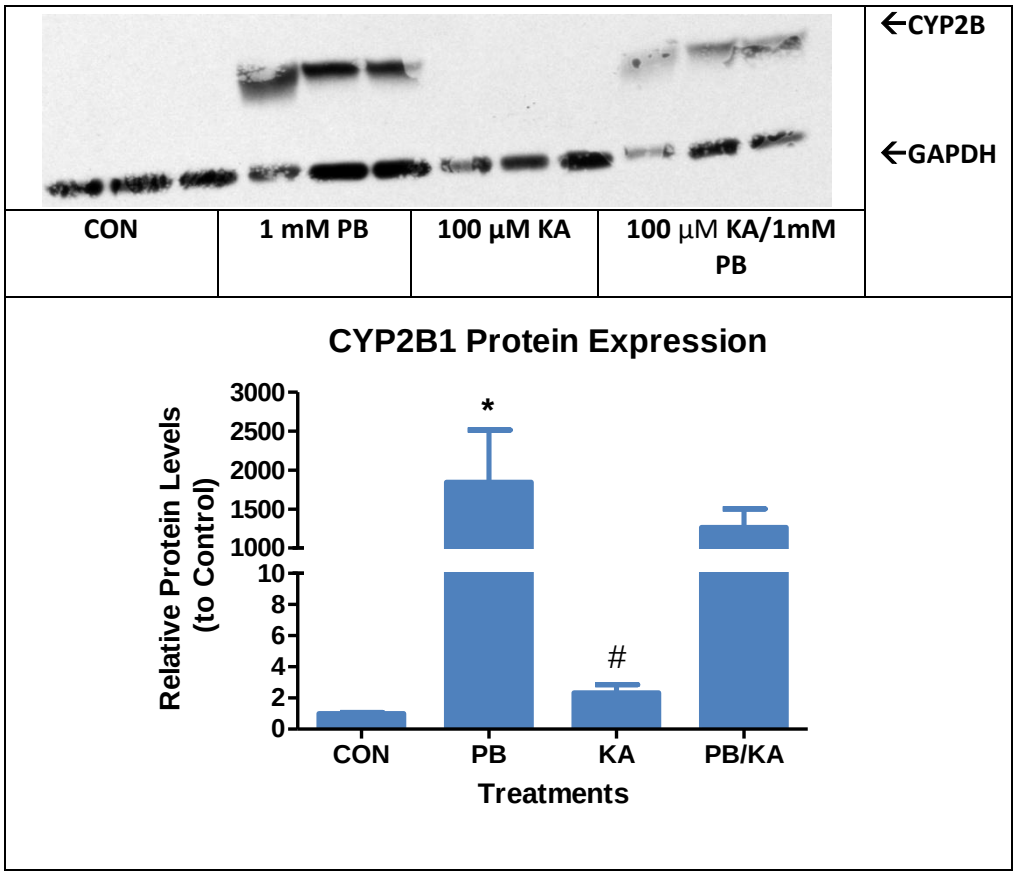


Figure 3.3i: Effect of PB and KA co-treatment on CYP2B protein expression. Primary rat hepatocytes were co-treated with PB and KA for 48 hours after which CYP2B protein levels were observed via western blot. Symbols * and # indicate significance from control and PB alone, respectively ($p<0.05$).

3.4.1 CYP3A2 mRNA Expression

The constitutive androstane receptor (CAR) also has the ability to regulate CYP3A genes by binding to the PXR response elements (PXREM) (Wei, et al., 2000). We wondered if the attenuation of CYP2B expression would also correspond with an attenuation of CYP3A with AHR and CAR agonist co-treatment. Using samples from the experiment in section 3.2, phenobarbital alone increased CYP3A2 in a time-dependent manner while TCPOBOP, a direct CAR agonist, did not increase CYP3A2 expression (**Figure 3.4a**). When comparing PB alone to PB and β-NF co-treatment there was an attenuation of CYP3A2 mRNA expression at all time points with a significant decrease at the 48 hour time point.

To further see the effect of PB and AHR agonist co-treatment on CYP3A2 expression combinational studies with PB and three AHR agonists were performed. Although none of the treatment groups were statistically significant the following trends were observed. Co-treatment with PB and 3MC did not decrease CYP3A2 expression when compared to PB alone while co-treatment with PB and β-NF decreased PB expression when compared to PB alone. Finally, PB and KA co-treatment increased CYP3A2 expression. Note that 3MC, β-NF, and KA alone did not increase CYP3A2 expression (**Figure 3.4b**).

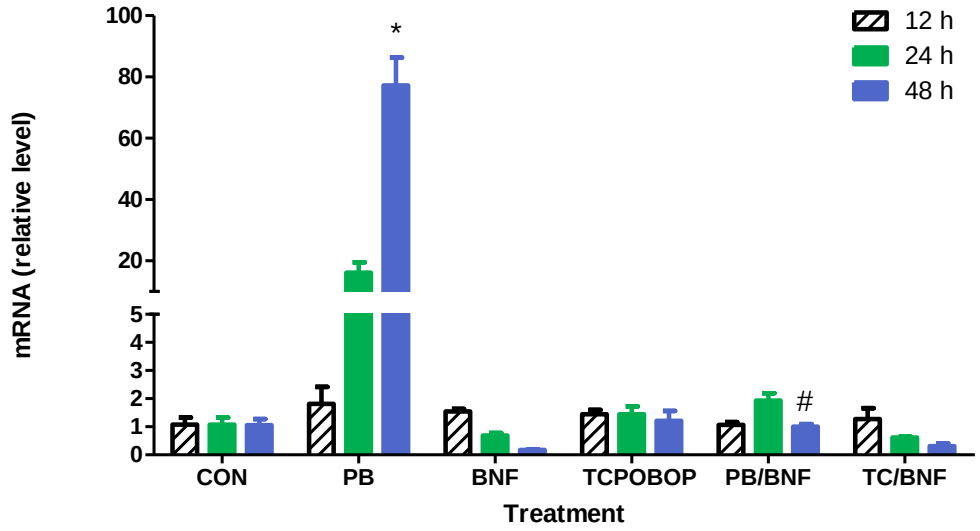


Figure 3.4a: Effect of simultaneous treatment with β-NF on CYP3A2 induction by CAR activators. Cells were harvested 12, 24, and 48 h after treatment with a CAR agonist with or without β-NF. CYP3A2 mRNA levels were determined by Real-Time RT-PCR. Symbols indicate significant from PB (#) and control (*), P<0.05.

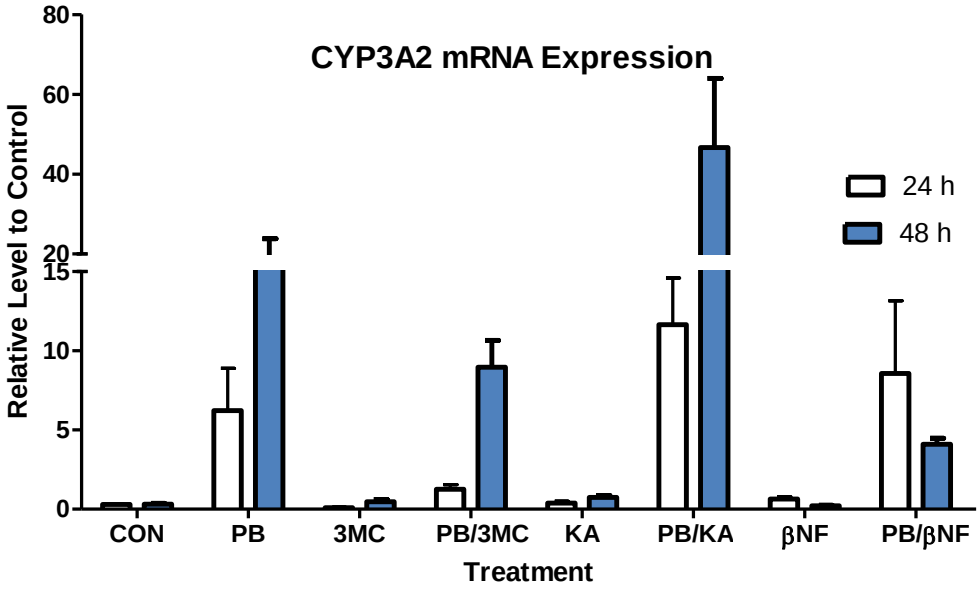


Figure 3.4b: Effect of treatment with AHR agonist and PB on CYP3A2 mRNA Expression. Cells were harvested 24 and 48 h after treatment with PB and AHR agonists. CYP3A2 mRNA levels were determined by Real-Time RT-PCR. (*Nothing significant*)

3.5 PXR and AHR: Effect of AHR agonist on CYP3A2 expression induced by PXR ligands

3.5.1 Dexamethasone The nuclear receptor PXR is a direct regulator of CYP3A in rat. It is also has the ability to cross-talk with CAR by binding to the PB-response elements that regulate CYP2B (Wei, Zhang, Dowhan, Han, & Moore, 2002). To further delineate whether or not the decrease in CYP3A2 expression with PB and some AHR agonists was mediated through a PXR: AHR interaction two efficacious rat PXR ligands, dexamethasone (DEX) and pregnenolone-16α-carbonitrile (PCN), were used (LeCluyse, 2001). Dexamethasone, a synthetic glucorticoid, has been used previously as an activator of rat PXR as well as human PXR and induces CYP3A gene expression (Hartley, et al., 2004) (Pascussi, Drocourt, Fabre, Maurel, & Vilarem, 2000). Some studies have shown that dexamethasone is capable of inducing murine CYP2B but this induction is dependent on the glucorticoid receptor (Audet-Walsh & Anderson, 2009); however, the induction of CYP3A is most sensitive to glucorticoids like dexamethasone (Monostory & Vereczkey, 1994) and reports of dexamethasone inducing rat CYP2B via PXR were not found.

Combinational studies with 20 μM DEX in combination with different concentrations of β-NF (1 μM-100 μM) were performed. After 48 hours of exposure to DEX with or without β-NF, CYP3A2 protein levels were observed. At all concentrations of β-NF with 20 μM DEX there was an increase in CYP3A2 when compared to DEX alone. Furthermore, concomitant treatment with β-NF and DEX did not decrease CYP3A2 protein expression at all (**Figure 3.5a**).

3.5.2 Pregnenolone-16α-carbonitrile (PCN)

Pregnenolone-16α-carbonitrile is a potent activator of rodent PXR but not human PXR (Jones, et al., 2000) and does not activate CAR (Wei et al., 2002).The concentration of PCN used, 10 μM, was taken from previous literature (Jones, et al., 2000). After 48 hours treatment of 10 μM PCN with or without β-NF (100 μM-1μM), CYP3A2 protein expression was observed, but there was no significant effect of β-NF (**Figure 3.5b**).

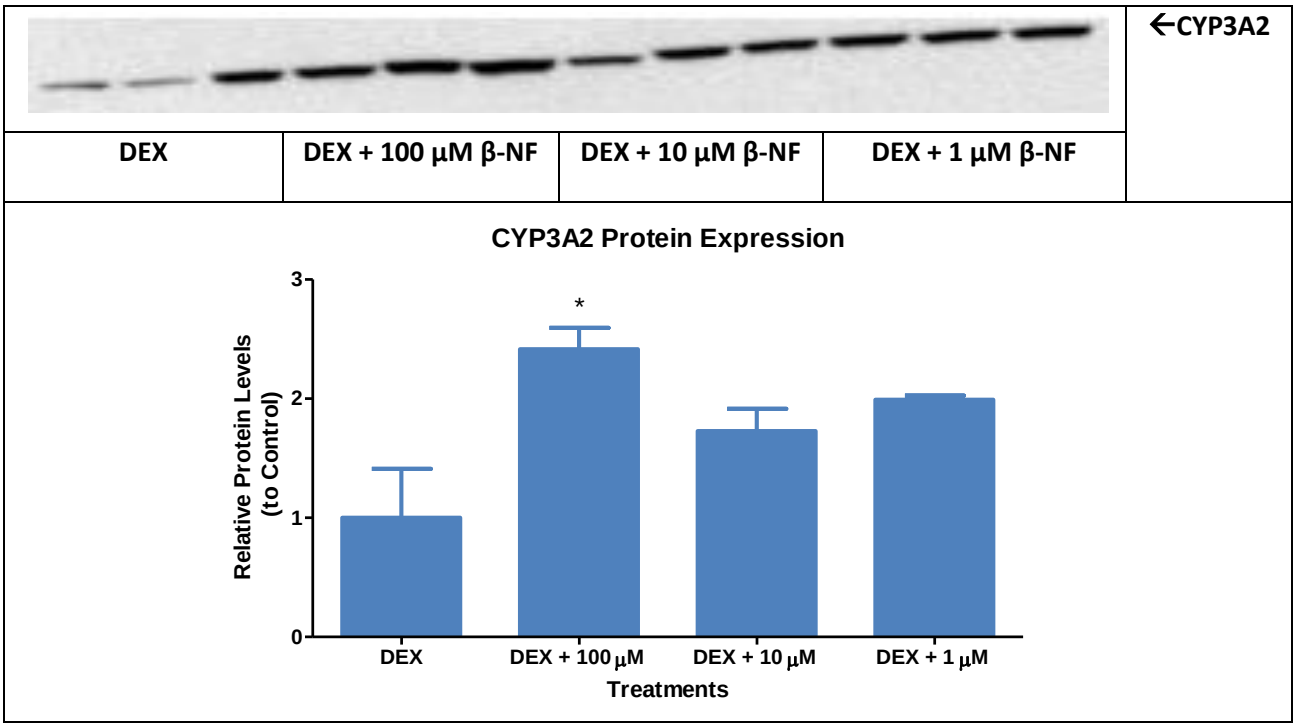


Figure 3.5a: **Effect of 20 µM dexamethasone (PXR ligand) with or without 10 µM β-NF (AHR ligand) on CYP3A2.** Concomitant addition of DEX with increasing concentration of β-NF increased CYP3A2 expression when compared to DEX alone with a significant increase at 100 µM β-NF. Co-treatment did not decrease CYP3A2 protein expression when compared to DEX alone. Symbol (*) indicates significance from DEX alone ($p<0.05$).

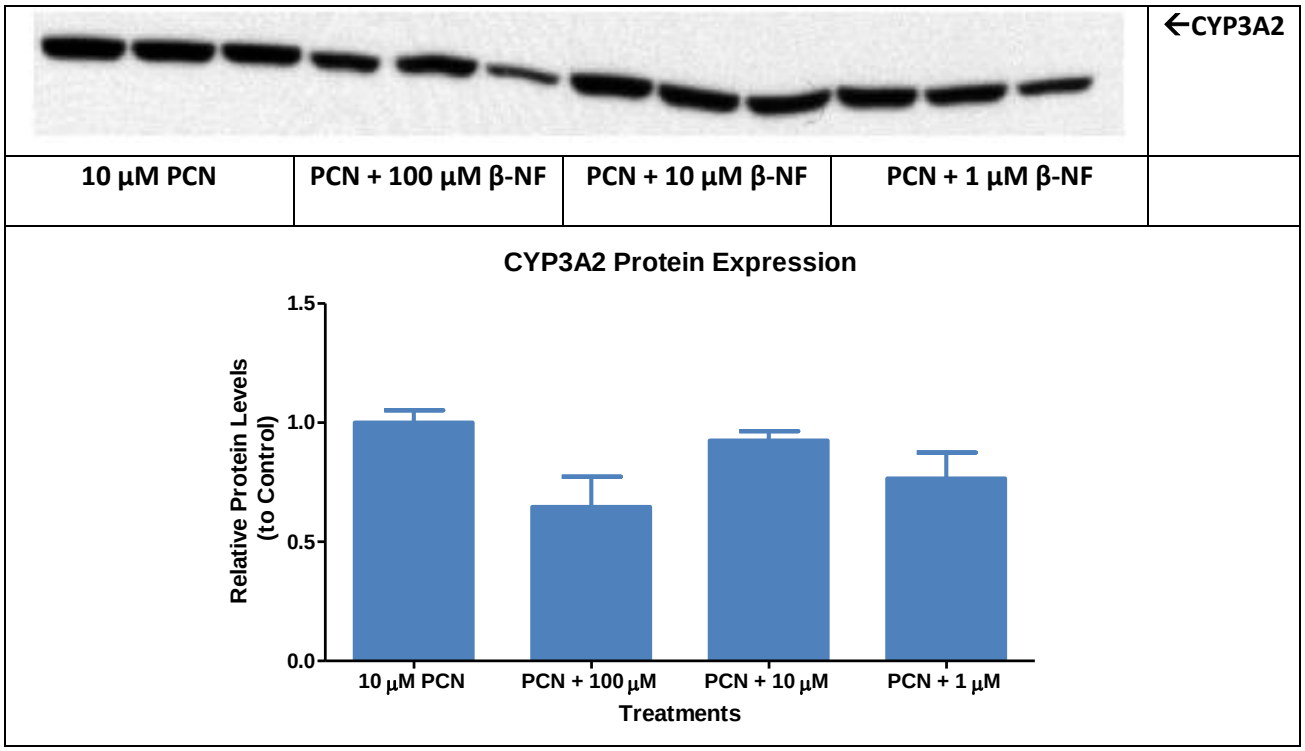


Figure 3.5b: **Effect of 10 µM PCN (PXR ligand) with or without 10 µM β-NF (AHR ligand) on CYP3A2.** Concomitant addition of PCN with increasing concentration of β-NF did not significantly decrease CYP3A2 protein expression. (Nothing Significant)

3.6 CAR Translocation

CYP2B regulation is dependent on CAR's translocation from the cytoplasm to the nucleus (Kawamoto T. , Sueyoshi, Zelko, Moore, Washburn, & Negishi, 1999) and this translocation has been shown to be inhibited by other compounds such as polyunsaturated fatty acids and decosahexaenoic acid (Li, Lii, Liu, Yang, & Chen, 2006) (Li, Lii, Liu, Yang, & Chen, 2007). In this vein, it was hypothesized that co-treatment with AHR agonist and phenobarbital may inhibit CAR's translocation to the nucleus thus attenuating CYP2B gene expression. Using an adapted protocol, as described in Materials and Methods, the nuclear and cytoplasmic fractions of rat hepatocytes were separated and CAR protein levels were probed using a commercially available anti-CAR antibody.

The CAR protein appeared around ~60-70 kDa. For cytosolic CAR, the CAR protein levels decreased for the PB alone group compared to the control while the other treatment groups had similar expression compared to the control (**Figure 3.6A**). β-NF treatment reduced nuclear CAR levels compared to the other treatment groups. Levels of nuclear CAR remained the same in the other three groups (**Figure 3.6B**).

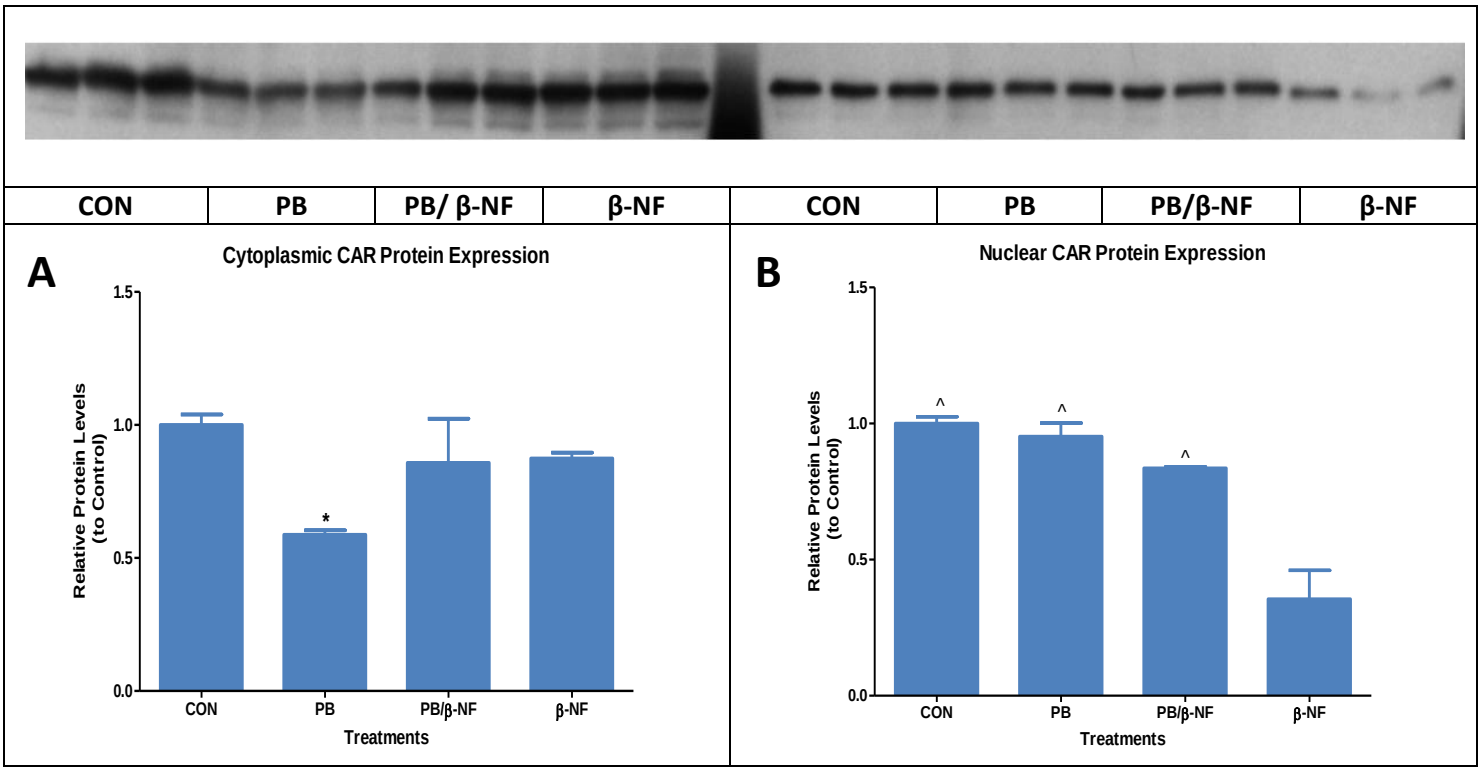
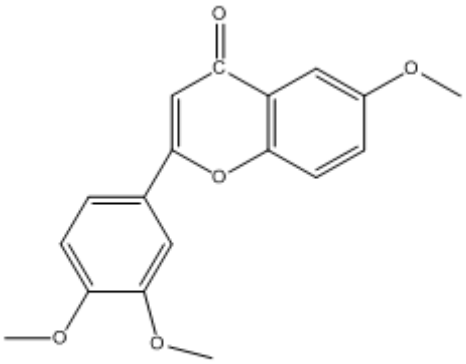


Figure 3.6: PB-dependent CAR translocation with β-NF co-treatment. Primary rat hepatocytes were treated with 1 mM PB, 10 µM β-NF, and a concomitant treatment with 1 mM PB and 10µM β-NF for 48 hours. **(A**) For cytoplasmic CAR levels, PB alone treatments decreased CAR expression in comparison to control. (**B**) For the nuclear CAR levels, β-NF decreased CAR levels. Symbols indicate significance from control (*) and from β-NF (^) ($p<0.05$).

3.7 Chemical Inhibition of AHR with TMF

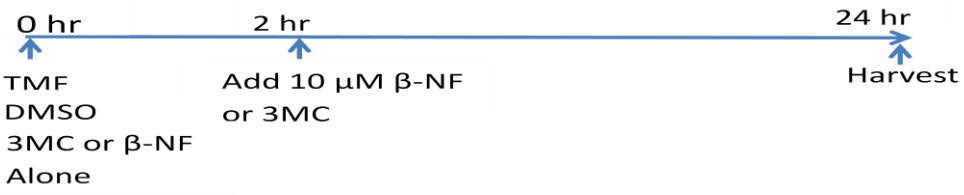


6,2,4-trimethoxyflavone

Figure 3.7a: 6,2,4-trimethoxyflavone (TMF) an antagonist of AHR.

To probe the requirement for AHR in the observed effects, a selective antagonist of AHR was selected. 6, 2, 4-trimethoxyflavone (TMF) (**Figure 3.7a**) was reported to be a potent antagonist of AHR with no partial agonist activity by Murray and colleagues (2010). TMF has the ability to compete with AHR agonists such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and benzo[a]pyrene and subsequently diminishes AHR/ARNT binding to DNA (Murray I. , et al., 2010). Inhibiting AHR would help to determine if the effects of β-NF and 3-MC that we observed were truly AHR dependent.

Primary rat hepatocytes were pre-incubated with increasing concentrations of TMF (10 nM-100 µM) alone for two hours. Then cells were exposed to additional β-NF (10 µM) or 3MC (10 µM) for an additional 22 hours and (**Schematic 3.7**). AHR activation was observed by measuring the levels of CYP1A2 mRNA. TMF did not inhibit CYP1A2 induction by β-NF or 3MC. (**Figure 3.7b, c**). Therefore, TMF is probably a ligand-specific or poor antagonist



Scheme 3.7: Experimental Design for AHR antagonist (TMF) coupled with AHR agonist (3MC or β-NF).

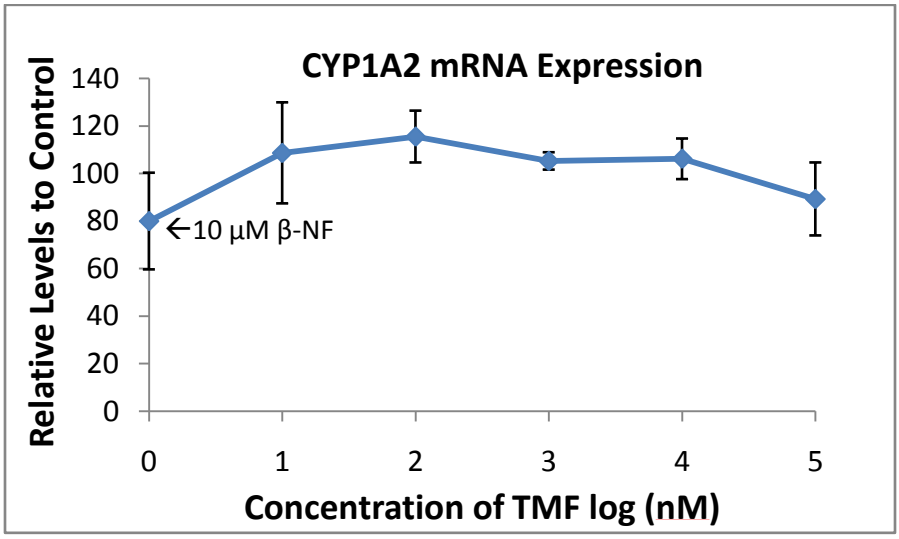


Figure 3.7b: Effect of TMF and β-NF co-treatment on CYP1A2 mRNA expression. TMF did not antagonize β-NF induction of CYP1A2 via AHR. (*Nothing Significant)*

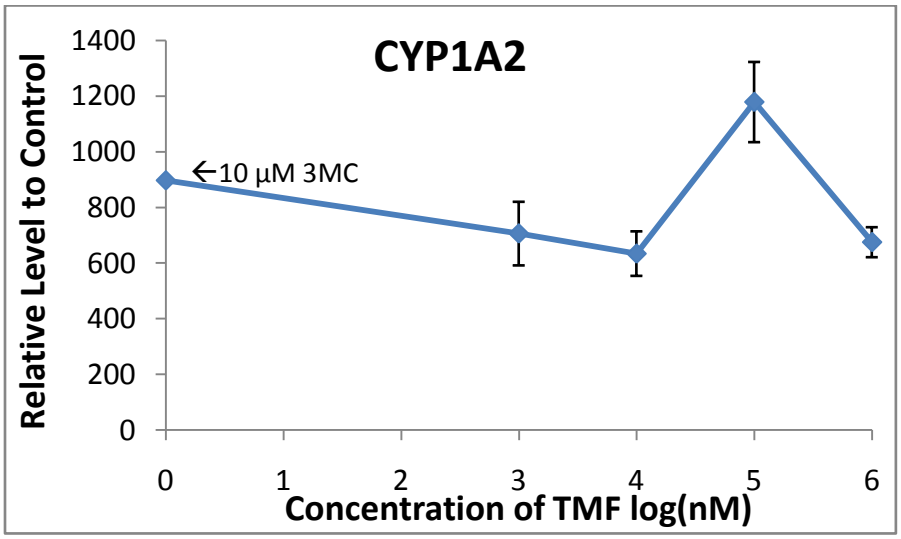


Figure 3.7c: Effect of TMF and 3MC co-treatment on CYP1A2 mRNA expression. TMF antagonized 3MC-depdent induction of CYP1A2 at the 1 µM and 10 µM concentrations. (*Nothing Significant)*

4. Discussion and Conclusions

The primary findings of this work are as follows: **1.** Activation of the AHR coupled with PB-dependent induction of CYP2B via CAR down-regulates CYP2B expression, suggesting a possible cross-talk between the AHR and CAR receptors. Not only did this down-regulation of CYP2B occur at a transcriptional level, but it was also observed with two structurally different AHR agonists, 3MC and β-NF. **2**. Combinational studies with PXR and AHR agonist suggested that a PXR: AHR interaction did not play a role in the down-regulation of CYP2B. More importantly, AHR does not repress CYP3A2 induction via PXR specific ligands, DEX and PCN. **3.** On the other hand, efforts to chemically inhibit AHR activation via an AHR antagonist were not successful suggesting that the antagonist used, 6, 2, 4-trimethoxyflavone, shows agonist-specific behavior. **4.** Furthermore, we present an attempt to investigate the translocation of CAR with CAR and AHR co-treatments; however, results from this investigation are not clear. **5.** Future work will have to be done to elucidate the mechanism by which β-NF induction of CYP1A2 via AHR antagonizes the PB-dependent induction of CYP2B via CAR.

1. PB-dependent induction of CYP2B via CAR was down-regulated by AHR activation. Furthermore, the down-regulation of CYP2B with AHR agonists and PB co-treatments was correlated with an increase in CYP1A2 levels, strongly suggesting an AHR: CAR cross-talk phenomenon. So far there has been only one report suggesting a possible CAR and AHR interaction. Patel and colleagues reported that in mouse liver, AHR activation *in vivo* led to a subsequent increase in CAR mRNA levels and CAR target genes (Patel, Hollingshead, Omiecinski, & Perdew, 2007). They also showed that this up-regulation in CAR mRNA and cyp2b10 was AHR dependent by using AHR knockout mice (Patel, Hollingshead, Omiecinski, & Perdew, 2007). This discrepancy between our results and Patel's can be attributed to species-specific differences. On the other hand, both studies showed β-NF increasing CYP2B levels with an increase seen after 6 hours in Patel's study and transient increase seen at 12 hours in our study. Finally, it is interesting to note that in our study after 48 hours exposure to 3MC and β-NF

the basal levels of expression for CYP2B were down-regulated suggesting that AHR activation can also act upon the constitutive activity of CAR or that β-NF can suppress CYP2B1 via a CAR-independent mechanism. Studying other CAR-regulated genes is warranted.

While the exogenous ligands 3MC and β-NF were able to down-regulate PB-dependent induction of CYP2B, the endogenous ligand kynurenic acid (KA) effects were weak. AHR is considered an orphan nuclear receptor because at present there are no known physiological activating ligands for AHR (Waxman, 1999). Many reports have shown tryptophan derivatives interacting with AHR either by inducing or inhibiting its target genes (Diani-Moore, et al., 2011) (Wincent, et al., 2008). However, these endogenous compounds are often at low levels and lack the potency needed to make them major regulators of AHR signaling (Adachi, et al., 2001). Kynurenic acid, a product of tryptophan oxidation pathway, was reported as a potent endogenous ligand of human AHR with the ability to induce CYP1A1/2 metabolism in primary human hepatocytes (DiNatale B. C., et al., 2010). However, in our experiments KA did not significantly induce CYP1A2. DiNatale and colleges exposed human hepatocytes to KA for a maximum of 18 hours (DiNatale B. C., et al., 2010) while in our study rat hepatocytes were exposed to KA for 24 or 48 hours. Furthermore, the induction of CYP1A1/2 seen in DiNatale's was poor compared to TCDD. Long exposure to KA coupled with poor induction of CYP1A suggests that KA is being rapidly metabolized in hepatocytes. Other endogenous AHR ligands such as indirubin have been reported to be rapidly metabolized *in vivo* despite their potency (Adachi, et al., 2001). Nevertheless, in the future other endogenous AHR ligands should be used to see if the attenuation of PB-dependent induction of CYP2B can also be inhibited by endogenous AHR ligands. In order to maintain AHR activation and to avoid the rapid metabolism of these endogenous ligands, the levels of endogenous ligands should be maintained with frequent replenishment without compromising cell viability.

2. To determine the extent to which a plausible PXR: AHR interaction could play a role in the attenuation of CYP3A during exposure to PB and AHR agonists we treated primary rat hepatocytes with

PXR specific ligands and β-NF. We found that the activation of AHR by β-NF did not inhibit PXR's ability to transcribe CYP3A. PXR primarily regulates the transcription ofCYP3A while CAR primarily regulates the transcription of CYP2B. However, CAR and PXR are known to cross-talk by binding to each other's response elements. Based on our experiment and previous studies it is more than likely that the decrease in CYP3A2 seen in some experiments was due to a CAR: AHR effect and not a PXR: AHR effect. First, PB is a weak PXR agonist and an increase in CYP3A expression is more than likely due to CAR's ability to bind XRE within the 3A promoter (Wei, et al. 2002). Similarly, DEX and PCN are specific PXR ligands that do not induce CYP2B through PXR and are not known to activate CAR (Wei, et al. 2002) (Jones, et al, 2000). Although, we did not look at CYP2B expression with the PXR ligands, the potential for PXR to induce CYP2B using PXR specific ligands is not likely. Finally, while other studies have shown that activation of AHR by 3MC leads to a decrease in CYP3A2 (Riddick & Jones, 1996) these studies were *in vivo* and did not use a PXR agonist concomitantly.

3. To observe if AHR activation was required for the observed phenomenon, an AHR antagonist TMF was used. TMF was first reported as an AHR antagonist with no partial agonist activity (Murray I. A., et al., 2010). However, after pre-incubation studies with TMF followed by treatment with β-NF or 3MC, TMF did not antagonize CYP1A2 induction. This effect is likely due to TMF's selective antagonist activity (Zhao, DeGroot, Hayashi, He, & Denison, 2010). Moreover, in a study using reporter gene expression assays, TMF inhibited TCDD-dependent induction but only partially inhibited β-NF induction suggesting that TMF is a selective inhibitor of halogenated aromatic hydrocarbons (HAHs). Furthermore, TMF failed to inhibit the ability of β-NF to stimulate AHR binding (Zhao, DeGroot, Hayashi, He, & Denison, 2010). Other AHR antagonists, such as CH223191, have also shown ligand-selective antagonism against TCDD but not β-NF or 3MC (Kim, et al., 2006) (Zhao, DeGroot, Hayashi, He, & Denison, 2010).

Future studies should attempt to use other AHR antagonists such as resveratrol (Casper, et al., 1999), α-napthoflavone (Santostefano, Merchant, Arellano, Morrison, Denison, & Safe, 1993) or 3', 4'-

dimethoxyflavone (Lee & Safe, 2000). However, many AHR antagonists show partial agonist activity or have AHR-independent activity (Murray I. A., et al., 2010). Until an AHR antagonist that is both specific against β-NF or 3MC and does not show partial agonist activity is available, it might be beneficial to inhibit AHR activation using genetic modifications such as AHR knockout rats or small interfering RNA (siRNA). However, AHR knockout rats are not readily available and most AHR knock out models are in mice not rat.

4. CAR translocation is a crucial step for the CAR mediated transcription of CYP2B (Kawamoto et. al, 1999) that is dependent on the phosphorylation of a threonine 38 (Mutoh, et al., 2009). We hypothesized that AHR activation could potentially inhibit CAR's translocation to the nucleus, subsequently decreasing CYP2B induction. We developed a method to probe cytoplasmic CAR versus nuclear CAR, adapted from Li et al. 2007 and observed bands for CAR protein at ~70 kDa. The expected molecular weight of CAR is approximately 46 kDa (Santa Cruz Biotechnology). Perhaps, the observed CAR was due to splice variants. Splice variants have been observed for both the human and rat CAR (Auerbach S.S., et al., 2003) (Kanno Y., et al., 2005). However, variants were not observed at ~70kDa. On the other hand, this method for identifying CAR levels is still preliminary and there is room for improvement. First, the use of an appropriate control such as an anti-histone antibody will help to determine the purity of the nuclear extracts and can also be used as a loading control. Secondly, a shorter exposure time with each agonist is needed to see the immediate effect of co-treatment on CAR translocation. Previous studies that monitored CAR translocation did not exceed the 24 hour time point (Li et. al, 2007) (Kawamoto T. , Sueyoshi, Zelko, Moore, Washburn, & Negishi, 1999) (Koike, Moore, & Negishi, 2005) with some observing CAR translocation in one hour (Kawamoto T. , Sueyoshi, Zelko, Moore, Washburn, & Negishi, 1999). In our experiment the levels of nuclear CAR were the same across treatment groups suggesting that because of the long exposure time (48 hours), CAR is being cycled out of the nucleus or being tagged for degradation. However, PB treatments alone showed a time-

dependent induction of CYP2B mRNA that persisted until 48 hours which seems to negate the speculation of CAR being cycled out of the nucleus. On the other hand, it is quite possible that AHR agonists do not inhibit CAR's translocation to the nucleus put perhaps inhibit the transcription of the CYP2B genes within the nucleus itself. Therefore, the reason why CAR nuclear levels did not change for the different treatment groups is not clear.

5. Future work should elucidate the mechanism of AHR antagonism of PB-dependent induction of CYP2B. Plausible mechanisms of how AHR can inhibit PB-dependent activation of CYP2B via CAR are summarized within **Figure 5 (a)** If AHR agonists could increase intracellular cAMP levels, this would decrease the PB-mediated induction of CYP2B (Sidhu & Omiecinski, 1995). High cAMP levels have been shown to inhibit the PB-mediated induction of CYP2B without inhibiting β-NF -mediated induction of CYP1A1/2 (Sidhu & Omiecinski, 1995). cAMP level could easily be observed using commercially available cAMP assay kits. However, there are conflicting reports of the role of cAMP in AHR signaling. In primary rat hepatocytes, an increase in cAMP did not inhibit β-NF mediated induction of CYP1A1/2 (Sidhu & Omiecinski, 1995), but in other cell types cAMP impairs AHR signaling (Oesch-Bartlomowicz & Oesch, 2009). **(b)** AHR inhibits the dephosphorylation of threonine 38 by inhibiting protein phosphatase 2A (Yoshinari, Kobayashi, Moore, Kawamoto, & Negishi, 2003), interfering with AMPK activation (Rencurel, et al., 2006), or dephosphorylating ERK ½ (Osabe & Negishi, 2011). CAR translocation is a crucial step for CYP2B gene activation (Mutoh, et al., 2009). If activated AHR were able to occlude CAR from traveling into the nucleus, then there would be a decrease in CYP2B transcription. **(c)** AHR agonists activate some factor or protein X that binds to the CAR cytoplasmic complex keeping CAR sequestered in the cytoplasm. Similarly, if some unknown protein inhibited CAR translocation there would be an overall decrease in CYP2B expression. **(d)** Activated AHR binds to the CYP2B promoter or within the CYP2B gene blocking the CAR transcriptional machinery. There is evidence that the activated AHR can bind to an inhibitory xenobiotic response element (iXRE) that stops the formation of a functionally active estrogen

receptor (ER)-Sp1 complex (Pocar, Fischer, Klonisch, & Homback-Klonisch, 2005) (Wang, Samudio, & Safe, 2001). The same could be true for AHR and CAR with the AHR binding and having repressive effects. A preliminary search of AHR binding sites within the CYP2B1 promoter did not find any AHR binding sites. However, chromatin immunoprecipitation (ChIP) assays after co-treatment with AHR agonist and PB could help to determine the binding sites of AHR and CAR differentially. **(e)** The activated AHR binds DNA outside of the normal CYP2B regulatory sequences and acts as repressor for CYP2B. Because the repressive effect of AHR also happens in a PB-independent manner, it is quite possible that AHR can bind outside of the CYP2B gene and act as a repressor that decreases CYP2B transcription. **(f)** Short heterodimer partners (SHPs) bind to CAR repressing the transcription of CYP2B.SHPs are orphan nuclear receptors that lack a DNA binding domain that can bind to other NR inhibiting the transcription of target genes (Klinge, et al., 2001). Because SHPs have been shown to inhibit both the transcriptional activity of CAR (Kemper, Kemper, & Bae, 2004) and AHR (Klinge, et al., 2001) it is not clear how SHPs would differentially inhibit CAR with AHR agonist and CAR agonist co-treatment. **(g)** Finally, squelching of CAR co-activators by AHR may suppress CYP2B transcription.

In our study we have shown that AHR activation coupled with PB-dependent activation of CAR leads to an attenuation of CYP2B. This attenuation of CYP2B was also seen in a PB-independent manner suggesting that AHR represses both the constitutively active and activated CAR. Furthermore, we have shown that this attenuation is an AHR: CAR interaction and not an AHR: PXR interaction. Attempts to chemically inhibit AHR were not successful; however future experiment will involve knocking down the AHR receptor. Finally, we have begun to develop a way to track the translocation of CAR with AHR agonist and PB co-treatments in order to determine if AHR inhibits CAR translocation.

Our work has huge implications for drug-drug interactions—for example, for patients taking anti-epileptics such as PB in conjunction with AHR agonists such as chemo-preventative medications or omeprazole. Similarly, adverse effects affect could happen for those taking drugs that activate CAR while

smoking. Overall, an understanding of how nuclear factors interact with each other will enhance our understanding of Cytochrome P450 regulation and thus drug metabolism.

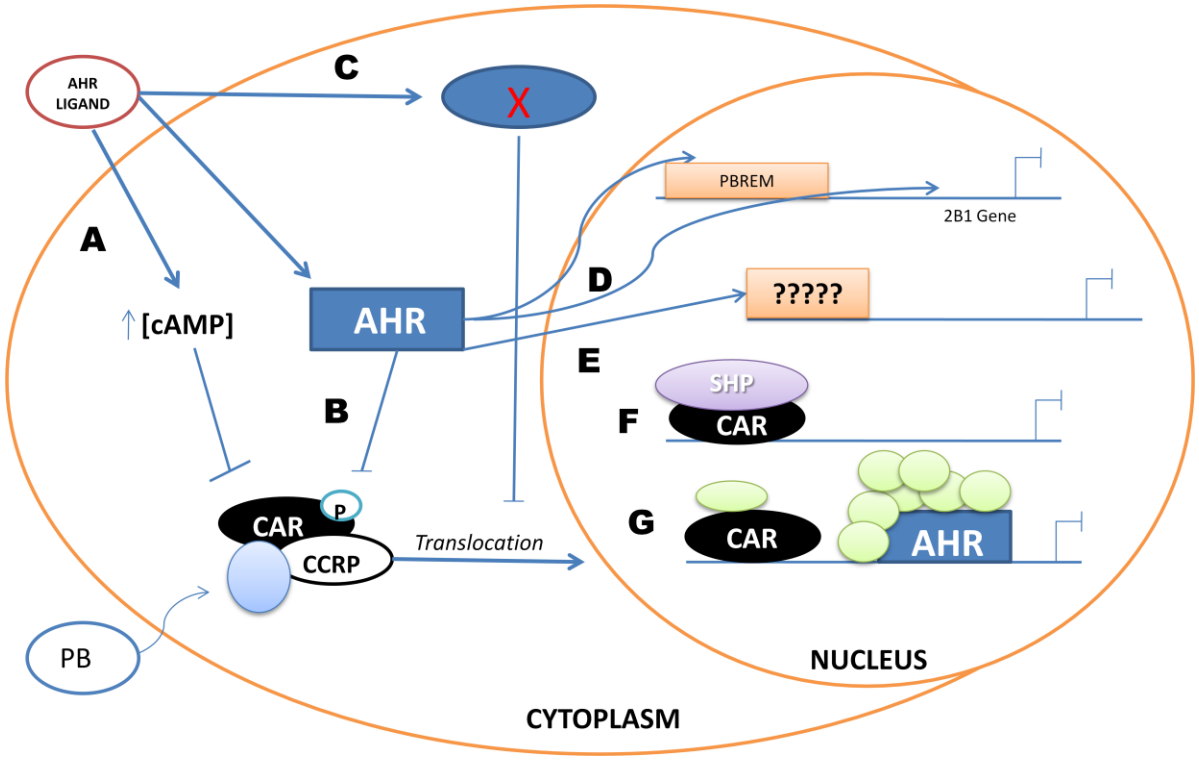


Figure 4: Plausible Mechanism for attenuation of CYP2B.

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